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THE SECOND REYNOLD A. SPAETH MEMORIAL LECTURE[1]

Protein Synthesis

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Department of Biochemistry, University of Toronto, Toronto, Canada

It is not necessary to elaborate on the importance of an understanding of the mechanisms involved in the enzymatic synthesis of proteins, because it is perhaps the most fundamental characteristic of living matter, just as protein itself is the most characteristic constituent of protoplasm.

We know something of the intermediate and a good deal of the final products of degradation of the protein molecule, though we know little or nothing of the exact structure of the molecule itself.

In the higher organisms which, unlike the plants, have not the power to synthesize amino acids, material for the synthesis of the proteins required for growth and maintenance must be ingested in the form of proteins or amino acids. The proteins, before final absorption, must be broken down in the digestive tract into the constituent amino acids by means of enzymes, and these amino acids are built up in the body into the infinite variety of proteins characteristic of the various organs and tissues.

Now it is not difficult to conceive the possibility of building up this immense variety of different proteins from the twenty or so different amino acids presented to it. But the mechanism by which the differentiation is achieved is more difficult to imagine, and provides a fascinating field for speculation. It is possible, of course, but improbable, that a

[1] Delivered at the Marine Biological Laboratory, Woods Hole on August 19, 1930.

specific enzyme performs each synthesis. Experiments which I shall describe this evening provide at least a possible alternative.

The first part of the problem to be attacked was, of course, the synthesis of protein from its degradation products by means of enzymes. It was then necessary to prove that a real synthesis had been achieved. The investigation and control of the conditions concerned in the synthesis was next attempted, and finally the possibility was investigated of producing from one substrate, with a single enzyme, proteins of different composition.

Those of our own experiments to which I shall refer were performed in collaboration chiefly with Dr. H. Borsook, now of the California Institute of Technology, and I shall always look back with pleasure to the many hours which we spent together in these investigations.

I should explain, perhaps, at the outset that we were unable to obtain synthesis from anything less complicated than peptic digests of various proteins, although it is possible to obtain synthesis from such substrates with several enzymes. The experiments do not, therefore, reproduce an *actual biological* synthetic process. Investigations into the mechanism of peptic synthesis and its control are, however, at least very suggestive as to the probable mechanism of the biological synthesis.

First let me say a few words about the theoretical aspects of enzymic synthesis. In applying the law of mass action to hydrolysis in general, it is usual to write:

$$A + H_2O \rightleftharpoons \text{products}$$
$$e.g.\ \text{ester} + H_2O \rightleftharpoons \text{alcohol} + \text{acid}$$

and the equilibrium equation in this case becomes

$$\frac{(\text{alcohol})\ (\text{acid})}{(\text{ester})\ (\text{water})} = \frac{K_1}{K_2} = K.$$

In such a system it is necessary, in order to obtain synthesis, to diminish the concentration of water as far as possible, and it has been assumed that the synthetic processes taking place in living cells include a very efficient mechanism or mechanisms for accomplishing this, such as surface condensation or imbibition by colloids. In our first experiments with protein synthesis *in vitro* the attempt was made to produce such surface condensation by means of the artificial cells used by T. B. Robertson and E. Newton Harvey. Robertson, you remember, used them to illustrate the mechanics of cell division and Harvey to demonstrate permeability. We were not successful in obtaining synthesis by this means alone but we returned to a similar procedure in later experiments, with interesting results which I will try to describe towards the end of this lecture.

To return to the question of concentration of water. In the alcohol-acid-ester system the molecular concentrations of water and of the other reacting components are of somewhat the same order of magnitude, but in protein systems, at least *in vitro,* the molecular concentration of water, even in very concentrated solutions, must be enormously greater than that of the other reacting components; and if we had to consider concentrations in the same terms as for the alcohol-acid-ester system, it would be impossible to attain syntheses *in vitro* because the relatively large molecular concentration of water would always send the reaction in the direction of hydrolysis. This is one reason why, under ordinary conditions of protein hydrolysis, the reaction is always complete, *i.e.,* the equilibrium position appears to be, unlike the ester system, at 100 per cent hydrolysis.

But in the hydrolysis of proteins and also, to a lesser extent, of fats, we have another factor, which alters the picture and minimizes the relative importance of the molecular concentration of water. This factor is the formation from one molecule of substrate (protein or fat), of a number of molecules of hydrolysate (amino acids or glycerine and fatty acids, as the case may be).

Hydrolyses of this type are in a special category, because the formation of *many* molecules from *one* molecule leads to other consequences, which were, as far as I know, first pointed out by Benjamin Moore. I will very briefly review his suggestions.

Moore deduced on thermodynamical grounds that the conditions of equilibrium for the reaction $A \rightleftharpoons B$ may be expressed by the equation

$$P_2 = KP_1,$$

where P_2 and P_1 are terms proportional to the osmotic pressures of A and B respectively.

When, however, one molecule decomposes into two, *i.e.,* $A \rightleftharpoons 2B$, as in the hydrolysis of an ester, the equilibrium reaction may be expressed by the equation

$$P_2^2 = KP_1.$$

No term is inserted for the water which takes part in the reaction because its molar concentration remains practically constant.

For such simple reactions as $A \rightleftharpoons B$ the ratio of the concentrations at equilibrium of A and B is a constant, and is independent of the absolute concentration, as the expression $P_2 = KP_1$ indicates. Consequently dilution or concentration of the system does not alter the proportions of the components at equilibrium, but where one molecule decomposes into *more than one molecule,* the equilibrium ratio is not

constant and is not independent of the absolute concentrations. For example, if in the equilibrium reaction $P_2^2 = KP_1$, the concentration is doubled,

$$\text{then } P_1 \text{ becomes } 2P_1$$
$$\text{and } P_2^2 \text{ becomes } 2P_2^2,$$

but since P_2 is the $^2\sqrt{\ }$ of the left-hand term,

$$P_2 \text{ becomes } \sqrt{2P_2^2} \text{ or } \sqrt{2} \times P_2$$

i.e., while P_1 has been doubled in concentration, P_2 has been only increased $\sqrt{2}$ or 1.4 times, so that under these conditions, when the concentration of substrate is doubled, the concentration of hydrolysate is less than doubled at equilibrium. In other words, the degree of hydrolysis is less, or in still other words, the tendency towards synthesis is greater. Conversely, on dilution, P_2 decreases in less ratio than P_1, that is, the degree of hydrolysis increases. This effect, in the case of substances like esters and disaccharides, is not very marked; it is more marked in the case of simple fats where, since the fat molecule decomposes into four molecules, the equilibrium reaction becomes

$$P_2^4 = KP_1,$$

and it is of course very marked in the case of proteins, where the value of the exponent is much higher.

The consequences of this relationship can be briefly stated as follows: In reactions in which one molecule is decomposed into a number of molecules the position of the equilibrium point depends on the absolute concentration of the solution. In concentrated solutions of such systems these reactions are incomplete,—there is a tendency towards reversion or synthesis. In dilute solutions these reactions tend to be complete and irreversible, *i.e.*, the tendency towards hydrolysis is great.

These effects of concentration and dilution apply with especial force in the case of proteins and of polysaccharides. They are independent of the effect of concentration on the molecular concentration of the water itself (which, as we have seen, cannot be practically reduced for proteins *in vitro* to proportions which would drive the reaction in the direction of synthesis), and they have two consequences: First they enable us to easily attain complete hydrolysis of proteins in dilute solutions, and second, they enable us to attain synthesis with great ease in concentrated solutions, eliminating the necessity for mechanisms for reducing the molecular concentration of water to dimensions commensurate with the molecular concentration of other components. Since

presumably the same considerations apply *in vivo,* we no longer need to postulate special biological mechanisms for effecting syntheses.

It can also be deduced on thermodynamical grounds, as Moore pointed out, that an increase in temperature should favour synthesis and a decrease should favour hydrolysis. The method for the synthesis of protein in a peptic digest of protein is then very simple: It consists simply in concentrating the digest, raising the temperature and adding pepsin. The [H^+] must also be controlled. Synthesis begins immediately and equilibrium is attained in a few hours. Under optimal conditions a yield of 50 per cent of protein has been obtained.

Since 1886, when the first synthesis of protein-like material was carried out by Danilewski, many workers have obtained synthesized protein. Sawjalow in 1901 gave to the protein precipitate the name plastein, and most of these protein precipitates have since been known by that name.

The first unequivocal proof that the precipitate obtained is a synthetic product was furnished in 1911 by Henriques and Gjaldbäk, who demonstrated that plastein formation is accompanied by a decrease in free animo-nitrogen, the converse of the common method of following protein hydrolysis by estimating the increase in free amino-nitrogen. We confirmed these results and it can be shown that the free COOH groups also decrease in a digest on plastein formation, and, as Rona has shown, exactly parallel the reduction in free amino groups.

The plasteins may be hydrolyzed by pepsin in the same manner as native proteins, yielding proteoses and peptones. Plastein, like native protein, is precipitated from its solution by trichloroacetic acid, which will not precipitate gelatin nor the proteoses, so that its complexity must be of the order of *native* protein. It gives, of course, all the usual protein reactions. Perhaps the best proof of synthesis is obtained from a fractional analysis which we made of digests where different amounts of plastein had been synthesized. We found that all fractions of the digest contribute to the formation of plastein.

There is no doubt then that these plasteins represent synthesized protein-like material. Their identity has been doubted by many because they apparently differ in physical properties (more especially in their insolubility) from the native proteins or, particularly, the proteins from which the digest is prepared. As a matter of fact their insolubility appears to be due to a denaturation which results from the conditions in which they are formed. Albumin and other soluble proteins under similar conditions, that is, in the presence of a concentrated digest at the optimum pH of 4.0, are rendered as insoluble as plastein.

We have then the first part of the simple problem completed. We

have synthesized protein and we have satisfied ourselves that the product is indeed protein and that it is synthesized from all the principal components, *i.e.*, that the proteose, peptone and sub-peptone constituents all contribute to the rebuilding of a protein or protein-like substance.

The next part of the problem is the investigation of the conditions under which the synthesis is obtained. This has yielded results of the greatest interest to us though I can discuss them only very briefly in this lecture.

In studying the effects of concentration on synthesis, we found the following relations. The limiting concentration below which no synthesis could be made to occur corresponds to the equivalent of about eight per cent of protein. Synthesis takes place at all concentrations greater than 8 per cent. With concentrations higher than 8 per cent there is a straight line relationship between the amount of protein synthesized and the concentration of material in solution: synthesis is in simple inverse proportion to the dilution. At high concentrations, however, that is, at concentrations which correspond to more than 40 per cent of protein, there is a rapid falling off, until at still higher concentrations no synthesis is obtained. We have no explanation for this falling off, but a similar phenomenon was observed by Armstrong and Gosney in the enzymatic synthesis of fats.

These results on the effect of concentration confirm Moore's prediction that the important factor in the enzymatic synthesis of proteins is the concentration of material in solution, and not the relative concentration of water. It can also be shown that as he predicted, the same holds true for hydrolysis: the tendency for hydrolysis diminishes as the concentration of protein increases, and in concentrated solutions of proteins, hydrolysis is incomplete no matter how much enzyme is used.

A study of the effect of the addition of proteins in each case shows that we are dealing with true equilibria in these reactions. Our experiments demonstrate that if previously synthesized protein is added to the system, synthesis is inhibited to an extent directly proportional to the amount of added protein, which also shows that the plastein takes part in the equilibrium as if it were in solution. Conversely it can be shown that addition of products to a digest in which hydrolysis of protein is going on inhibits hydrolysis to an extent proportional to the amount of products added.

I mentioned earlier that Moore had deduced on thermodynamical grounds that in reactions of the type of protein synthesis and hydrolysis, the effect of temperature on the equilibrium position is such that increase of temperature favours reversibility, *i.e.*, increase in temperature favours synthesis and decrease favours hydrolysis.

We were able to confirm this deduction by a very striking experiment. A solution in which hydrolysis was proceeding at a temperature between 20°–24° was adjusted to the optimum pH for synthesis. It was then divided into three portions. One was maintained at 20° and showed the further change in the direction of hydrolysis to be expected under the circumstances. The second portion was raised to 65° C. In ten minutes plastein began to form and in 30 minutes considerable protein had been synthesized. A third portion was boiled to destroy the enzyme and then maintained at 65° C. for 8 hours without the occurrence of any change except the faint coagulation produced on boiling.

In this experiment then we have a solution in which hydrolysis is proceeding at 20° C., and simply by raising the temperature we are able to induce synthesis. We find that the equilibrium amount of protein synthesized increases with increasing temperature up to 72° C., at which temperature the enzyme is destroyed; that is, the effect of increase of temperature is to move the equilibrium point more and more over to the protein side.

So far I have made hardly any reference to the effect of hydrogen ion concentration on peptic synthesis. As you could guess, it is a most important factor. Practically all the previous work on protein synthesis was performed before the importance of the $[H^+]$ in enzyme reactions had been realized, and before methods for its exact determination had been developed. The only determination, that of Henriques and Gjaldbäk, had placed the optimum for synthesis with the optimum for hydrolysis at a pH of 1.5. On many occasions we determined the optimum for synthesis to be at a much more alkaline reaction, *viz.*, pH 4.0. It has been shown by Oda that this optimum holds, regardless of the acid employed, and that it is unaffected by the presence of a great variety of salts.

We have tried to determine the mechanism by which the $[H^+]$ controls the extent of peptic synthesis. The following interesting fact was discovered: If we construct a curve, plotting the yields obtained at different $[H^+]$, and plotting the yields obtained as per cent with the yield at the optimum pH as 100 per cent, the curve resembles in form and in slope either the primary dissociation curve of a dibasic acid or the undissociated residue of an amphoteric electrolyte. It seems probable from this resemblance, or correspondence,—because the correspondence is remarkably exact,—that the amount of protein synthesized at any given $[H^+]$ is dependent on the degree of ionization of some component of the system. It can hardly be the ionization of the enzyme which is concerned, because, as Northrop has shown, pepsin exists as

a monovalent anion between pH 1–7, and changes in pH within that range do not affect the degree of dissociation. It might be an effect on the autodestruction of the enzyme, but at least within the range of pH 1–4 there is no autodestruction under the experimental conditions. One can only suppose that the result is to be interpreted as showing the relation between synthesis and ionization of the digest itself; but titration curves of the digest show that the ionization of the digest as a whole is not represented by these curves, and one is forced to the conclusion that since there is this remarkable correspondence between the experimental values and a theoretical dissociation curve, and since we have ruled out other possibilities, the protein synthesized at any given $[H^+]$ is dependent on the degree of ionization of one component of the digest or rather, of the substrate.

Another respect in which peptic synthesis behaves rather differently from other enzyme reactions is in the effect of varying enzyme concentration on the equilibrium position. It has been found by other observers, and confirmed by the writer, that the equilibrium amounts of protein synthesized are increased with increasing amounts of pepsin. This is, of course, an exception to the general law of enzyme action and catalysis, which always assumes that the equilibrium position is not affected by the concentration of the enzyme. The explanation of this effect of varying concentrations of enzyme is suggested by another series of experiments which we carried out on the effect of the presence of emulsions in the system on peptic synthesis. These are the experiments I referred to earlier as resembling the experiments where the substrate was concentrated at the surface of artificial cells. We found that certain emulsions, such as those formed when chloroform, benzene, benzaldehyde, etc., are shaken up with the digest, accelerated the attainment of equilibrium. Moreover, in the presence of some emulsions, such as benzene, toluene, benzoic acid and benzaldehyde, synthesis is effected even in the absence of enzyme.

Now we may consider that pepsin, being a colloid or in colloidal association, owes its action, in part at least, to properties resembling those possessed by these emulsifying agents. This action occurs by virtue of the presence of the interphase surface. The action of emulsifying agents further resembles that of pepsin in that increasing amounts, under certain conditions, also augment the yield of protein synthesized at equilibrium. This effect of concentration of enzyme or emulsifying agent on the equilibrium is consistent with thermodynamical conceptions, since the addition of an emulsifying agent means an increase in the interphase surface, and this increase in surface means in turn an increase in surface energy to the system because of the increased amount of

surface energy available. Thus, increasing the concentration of pepsin should have the same effect as that obtained when energy in any other form is added to the system, as, for example, by increasing temperature. We have already seen that increasing the temperature up to the limit of destruction of the enzyme has a similar effect. On this basis we can therefore explain the departure from the classical conception that the concentration of enzyme should have no effect on the amount synthesized at equilibrium.

There is another point of similarity in the action of pepsin and of emulsions. T. B. Robertson, in 1926, discussed the possibility that concentration of the reactive groups of amino acids by orientation at lipoid surfaces might account for the completeness of enzymatic synthesis in living tissues. As we have just seen, in certain cases synthesis can be effected even without enzyme by the emulsifying agent alone if it is an accelerator of synthesis. Some of these emulsifying agents, *e.g.,* benzene and toluol, are chemically inert compounds. They can hardly enter into chemical combinations with digest constituents, and they are readily separable again from the synthesized protein. Their effect is almost certainly due to a specific orientation of the components of the digest which participate in the synthesis. That this action is specific is shown by the fact that the chemical and physical properties of the proteins synthesized vary with the means employed in synthesis. The base-combining properties, the ratios of free amino and free carboxyl groups to total nitrogen, the hydrolysability by pepsin, all show marked differences which are reproducible.

The solubilities also show very marked differences, *e.g.,* proteins synthesized by pepsin with or without benzaldehyde are soluble only at alkalinities greater than pH 7.7; at 7.6 the proteins are precipitated almost completely.

Proteins synthesized with benzaldehyde in the absence of pepsin are not precipitated until the reaction reaches pH 6.0, and the benzene-synthesized protein is not precipitated until the pH reaches 5.0. At more acid reactions these proteins are also completely precipitated.

These results indicate that, presumably through variations in the orientation of the participating components of the digest, proteins of *different chemical composition* are synthesized. They suggest also a possible explanation for the specificity of the action of the enzymes themselves.

We have been able to work out other interesting suggestions from studies of the conditions of peptic synthesis, but time will not permit me to discuss these results.

It is unfortunate that we have had very little success with protein

synthesis from digests less complex than peptic digests, but we still hope to obtain synthesis from simpler substrates, and are continuing our studies.

Peptic synthesis, therefore, is at best only a possible model of the biological synthesis of proteins. However, it provides many interesting suggestions as to the mechanism of the biological process, and not the least important of these is the last discussed, namely, the suggestion that specific orientation on the colloidal interphases provided by the proteolytic enzymes and their associated colloids may best account for the ability of the organism to synthesize its many varieties of proteins from a common substrate.

STUDIES ON AMPHIBIAN METAMORPHOSIS

IX. Integumentary Specificity and Dermal Plicæ Formation in the Anuran, Rana pipiens

O. M. HELFF

(From the Department of Zoölogy, State University of Iowa, and the Department of Biology, University College, New York University)

Introduction

The dermal plicæ or folds of newly-metamorphosed *Rana pipiens*, as is well known, constitute a striking and characteristic development for this species. Although minor folds have been described, the most distinct plicæ are those of the dorso-lateral trunk and lateral upper jaw regions. The former consists of two folds, one on each side of the trunk, running from the dorsal-posterior border of the eye back to a point just lateral to the urostyle prominence. The latter likewise consists of two folds, one on each side of the upper jaw, running from a point just anterior and ventral to the external nares posterior on a level just ventral to the tympanic membrane to a point just dorsal to the origin of the fore-limb. The folds are usually about 0.5 mm. in width at this time and are white in color in sharp contrast to the black or dark-greenish coloration of the adjacent integument. Histologically, the folds are seen to be composed of integument having a much thickened stratum spongiosum in which are found mucous and unusually large poison glands. It is the concentration and abnormal size of these glands in this particular region which result in the formation of an actual ridge or fold as viewed externally.

The development of the dermal plicæ during metamorphosis may be roughly estimated by external observation. In the normal larva, just prior to the onset of involution, no indications of plicæ formation are visible. During the early stages of transformation, however, broad though very indistinct bands of integument are noticeable in the dermal plicæ regions. These are at first of a dull, grayish-white shade. Later, however, towards the close of metamorphosis, they become lighter in color, as the bands appear to narrow, and more sharply demarcated from the adjacent integument. Following larval transformation, the plicæ become increasingly whiter and very distinct, after which they undergo relatively little change during the early growth period of the young frog.

The embryology of the mucous and poison glands as they occur in amphibian integument has interested many workers and given rise to numerous conflicting results and interpretations. Practically all workers, however, agree that the glands have an ectodermal origin, being derived from the Malpighian layer of the epidermis. The point of difference seems to lie chiefly in whether or not the two types of glands have a common origin or arise from separate sources. Separate developments have been described by Schultz (1889) and Nirenstein (1908) in *Salamandra,* by Heidenhain (1893) and Nicoglu (1893) in *Triton,* by Engleman (1872) and Seeck (1891) in the frog, and by Dawson (1920) in *Necturus.* Common origins, which in some cases have regarded the mucous and poison glands as simply different developmental stages of one and the same gland, have been described by Ancel (1902) in *Salamandra,* by Fano (1903) in *Triton,* by Esterly (1904) in *Plethodon,* by Wenig (1913) and Calmels (1883) in the toad, and by Szczesny (1867), Junius (1896), and Weiss (1908 and 1915) in frogs.

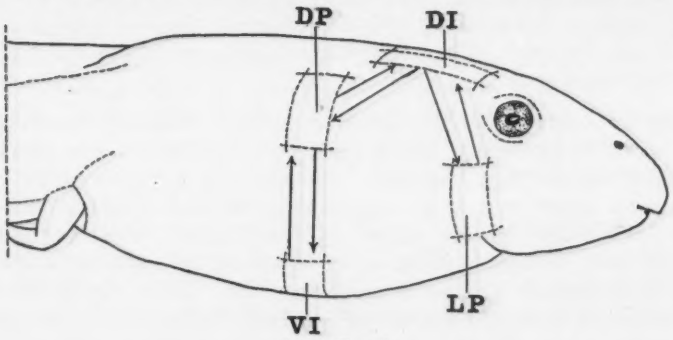
Although the present work has indicated certain points regarding the embryology of the acinous glands as they occur in *Rana pipiens,* the main object in view was to determine to just what extent various integuments of the larva are specific for glandular formation as found in dermal plicæ regions. Histologically, all integument of the larva, prior to the onset of metamorphosis, is similar in that there is an absence of acinous glands or of any indication of their future development. The question naturally arises, therefore, whether or not the integument of the dermal plicæ regions is hereditarily specific for the development of the glandular picture as it is found during larval involution. Conversely, the possibility suggested itself that all integument might be so stimulated to unusual glandular development, if located in the specific regions characterized by dermal plicæ formation during metamorphosis. The above points were subjected to analysis by making suitable integumentary transplantations. In general, these consisted of the autoplastic transplantation of larval integument from the dorso-lateral and lateral dermal plicæ regions to the back or belly, and vice versa. The development or non-development of dermal plicæ tissue in such transplants during larval involution could then be studied and information relating to integumentary specificity in this respect obtained.

The writer wishes to express his appreciation for the facilities tendered him during the summer of 1929 by the Iowa Lakeside Laboratory, Milford, Iowa, where the operations described in the present paper were made.

Materials and Methods

The stock used for all operations consisted of large *Rana pipiens* tadpoles collected in the vicinity of Spirit Lake, Iowa, during the months of June and July, 1929. Prior to the onset of metamorphosis the larvæ of this particular strain of *Rana pipiens* grow to the unusual size of 110–115 mm. total length. When collected and operated on, however, they averaged about 80 mm. in length, with hind limbs 8 to 12 mm. long. The selection of larvæ in a stage at least three weeks prior to the earliest signs of metamorphosis was essential, since preliminary sectioning of integument had shown that the acinous glands begin their development at about the beginning of the metamorphic period.

The various integumentary transplantations were made as follows: The larva was first anæsthetized in a 0.05 per cent aqueous solution of chloretone. The approximate regions of the integument in which dermal plicæ development later occurs were next located and a rectangular piece of skin, large enough to include a small amount of the adjacent integument above and below the dermal plicæ region, was cut and removed. The fact that all integument, including that of the dermal plicæ regions, is of the same macroscopic appearance, necessitates a knowledge of the approximate level of integument which will later develop into dermal plicæ, in order that the skin graft will include all of the potential plicæ integument. A similar sized piece of integument was now removed from either the mid-dorsal or mid-ventral region of the same larva and transplanted to the wound area left subsequent to



Text Fig. A

the removal of the dermal plicæ skin graft. The latter was now transplanted to the area exposed by the removal of the back or belly integument (text fig. *A*). Following this, the larva was placed in shallow water so as to expose the two grafted areas to the air, thereby hastening

the adhesion of the transplants. Following adhesion of the transplants, the larva was immersed in an individual aquarium and allowed to recover from the effects of the anæsthetic. Subsequent treatment included feeding with *Spirogyra* to induce growth and daily observations to record the onset of metamorphosis and the development of macroscopic signs of dermal plicæ formation in the various transplants during larval involution. Representative skin transplants were removed at various stages of metamorphosis in order to determine the development or non-development of glandular structures.

Results

Normal Histological Development of Dermal Plicæ Structures

Although the normal development of the acinous glands has been pictured and described by many authors in a variety of amphibians, relatively few have dealt with the formation of these glands as they occur in the dermal plicæ and none, with the possible exception of Massie (1894), has dealt with the conditions as found in *Rana pipiens*. It is assumed that Massie probably worked with *Rana pipiens*, although the species of frog tadpole used was not specifically stated. The exact mode of the various structural differentiations in relation to the results of skin transplantation as described in the present paper was not so important to determine as was a knowledge of the histological picture of the dermal plicæ typical of various stages of larval involution.

For convenience, three stages of glandular development in dermal plicæ regions corresponding with three stages of larval involution will be briefly described. These descriptions apply especially to the dorsolateral dermal plicæ.

Stage 1. *Larvæ within One Week of Onset of Metamorphosis.*—The larvæ are in all respects typical tadpoles. There are no macroscopic signs of dermal plicæ formation. Histologically, all layers of the integument appear normal as regards thickness and pigmentation. A few, widely-scattered, oval-shaped, embryonic gland "nests" are to be found partly embedded in the lower layers of the epidermis and invaginated into the stratum spongiosum. The "nests" appear to be composed of large, oval-shaped nuclei and cells having large nuclei with a minimum of cytoplasm. Mitotic figures are present in some of the cells. The "nests" all appear to resemble one another cytologically. It is clear that the component cells have been derived from the Malpighian layer of the epidermis, probably for the most part from the nuclei.

Stage 2. *Larvæ in Early Stage of Metamorphosis.*—The visible external metamorphic changes of the larva include growth of the hind-limbs and slight atrophy of the tail. Otherwise, the larva is still tadpole-like in most respects. Externally, the dermal plicæ are indicated by broad, indistinct bands of a grayish coloration. Histologically, the epidermis is unchanged in thickness but appears to be less pigmented. The stratum spongiosum is considerably thickened to accommodate the glands. The poison glands are fairly well developed and of a good size. The muscular and fibrous layers of the latter are present. Practically all have ducts developed. The inner epithelial layer is composed of cuboidal-shaped cells, while large granules occupy the central cavity of the gland. The glands are definitely poison glands. Small mucous glands are also found, some of which have acquired ducts. In addition, glandular "nests" are also found in the usual location, the inference being that they are developing mucous glands. The adjacent back integument now possesses a few, widely scattered gland "nests," which appear smaller than those described in Stage 1 dermal plicæ.

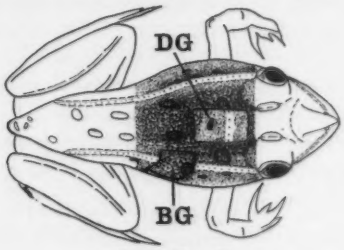
Stage 3. *Larvæ near End of Metamorphosis.*—The animals are typically frog-like, although the tympanic membranes are not as yet fully developed. The tail has atrophied to a small stump. The dermal plicæ appear narrower, distinctly marked off from the adjacent integument and grayish-white in color. Histologically, the epidermis has thickened and contains more layers than that of the adjacent back integument. The stratum spongiosum is especially thickened, while the melanophores in this layer and the epidermis appear to be considerably less evident. The integument as seen in cross-section is markedly convex along its epidermal surface. The glands are much larger than hitherto described. The poison glands show considerable variation in size, while the epithelial cells lining the base of the gland cavity are usually columnar in shape. The mucous glands are more numerous and larger than in Stage 2, while glandular "nests" may still be found, especially towards the edges of the dermal plicæ.

Further developments, following Stage 3, include the general whitening of the plicæ as viewed externally, while histologically the glands (especially the poison glands) increase still further in size and tend to crowd together to form the arrangement typical of the fully developed dermal plicæ.

Reciprocal Transplantation of Dermal Plicæ Integument with that of the Back and Belly

Series A.—This series consisted of reciprocal, dorso-lateral dermal plicæ and back skin transplantations (*DP* and *DI*, text fig. *A*). Fifty-

seven larvæ were operated on in all. During metamorphosis, it was soon evident that typical macroscopic signs of dermal plicæ formation were present in the grafts transplanted to the back. If the graft had been transplanted in the same orientation as it had previously occupied in relation to the anterio-posterior axis of the body, the dermal plicæ developed, parallelling the normal ones of the animal. If, however, the graft had been rotated 90° during transplantation, then the dermal plicæ developed at right angles to the normal ones of the animal. The narrowing and whitening of the dermal plicæ band of integument progressed at a uniform rate identical with the normal formation of the dorso-



TEXT FIG. B

lateral dermal plicæ on the same animal (*DG*, text fig. *B*). It was also observed that the general pigmentary changes occurring in the transplant were characteristic of the region of integument from which the graft had been secured. The back skin grafts transplanted to dermal plicæ regions underwent macroscopic changes in coloration, including the development of spots, and corresponding in all details to the pigmentary changes of the integument of the back. There were no external indications of dermal plicæ formation, not even where the edges of the grafts were in contact with the developing normal dorso-lateral dermal plicæ (*BG*, text fig. *B*). Following the complete resorption of the tail, the metamorphosed animals generally lived for about three weeks, at which time they died due, no doubt, to lack of proper food. Representative skin transplants were removed at this time and sectioned for histological study.

The microscopic appearance of dermal plicæ integumentary grafts transplanted to the back is represented in Fig. 1, Plate 1. It will be noted that typical glandular development and tissue thickening has occurred, corresponding in all details to the condition typical of normal dorso-lateral dermal plicæ at this stage of the animal's life. The various histological features are representative of a somewhat later stage, as previously described for Stage 3 of dermal plicæ development. The epidermal and stratum spongiosum layers are especially thickened, giv-

ing the external surface of the dermal plicæ integument a decidedly convex shape. The poison glands are larger than those described for Stage 3 and more closely crowded together. Usually five or six are found across the width of the plicæ. The mucous glands are relatively much smaller and fewer in number and occupy only the upper regions of the stratum spongiosum. The microscopic appearance of the back skin grafts transplanted to dorso-lateral dermal plicæ regions is represented in Fig. 2, Plate 1. Again the various histological structures correspond to the conditions typical of normal back integument at this time. The epidermis is normal in thickness, while the stratum spongiosum is but moderately thickened to accommodate the glandular development. Regarding the latter, the mucous glands appear to predominate both in size and number. In fact, the mucous glands are larger and more numerous at this time than is true of normal dorso-lateral dermal plicæ. Only a few, small, widely-scattered poison glands are present, which have probably just begun their development (*PG*, Fig. 2, Plate 1). It should be emphasized, however, that this same histological picture is typical of normal back integument at this time.

Series B.—Series B consisted of reciprocal, dorso-lateral dermal plicæ and belly skin transplantations (*DP* and *VI*, text fig. *A*). Ten larvæ were operated on in all. The dorso-lateral dermal plicæ integumentary grafts developed normal dermal plicæ during metamorphosis which, together with the characteristic pigmentation pattern of the adjacent transplanted back skin, presented a striking contrast to the surrounding white belly skin (Fig. 5, Plate 1). Histologically, the same glandular and integumentary conditions were found as already described for this type of graft when transplanted to the back (see Series A, above). The belly skin transplants were typically pure white in color when transplanted to the dorso-lateral dermal plicæ regions. This lack of melanophore pigmentation and spotting was maintained during subsequent larval involution and likewise presented a strong contrast as compared with the darkly pigmented adjacent skin of the back and side (Fig. 6, Plate 1). The histological appearance of such grafts, at a time when the normal dermal plicæ were well formed, is represented in Fig. 3, Plate 1. Compared with back skin grafts transplanted to this region, the belly skin transplants were somewhat thinner as a whole, while the mucous glands were correspondingly smaller though more numerous. The poison glands were very small and scarce. In general, however, it may be said that the microscopic appearance was identical with the normal integument of the belly at this time.

Series C.—This series consisted of reciprocal, lateral (upper jaw) dermal plicæ and back skin transplantations (*LP* and *DI*, text fig. *A*).

In all, twenty larvæ were operated on. The development of typical macroscopic signs of dermal plicæ formation were found to occur during involution in the transplants made to the back. The appearance was quite similar to the dorso-lateral transplants to the back as illustrated in text fig. *B*, except that the whitening of the integument was slightly delayed and the width of the plicæ bands somewhat narrower. This, however, is typical of normal lateral plicæ development. The back skin grafts transplanted to lateral dermal plicæ regions, like the back skin transplants of Series A, failed to exhibit visible macroscopic signs of plicæ formation during larval involution. Histologically, the appearance of these transplants was also similar to that described for the back skin transplants of Series A and pictured in Fig. 2, Plate 1. The microscopic appearance of the lateral dermal plicæ transplants on the back presented several differences as compared with the dorso-lateral dermal plicæ transplants described in Series A and pictured in Fig. 1, Plate 1. Figure 4, Plate 1, represents a typical section taken through one of these transplants at the usual time,—some three weeks following the completion of larval involution. Perhaps the greatest difference observed was in the relatively greater abundance and size of the mucous glands. The poison glands, on the other hand, were smaller and less numerous than in the dorso-lateral dermal plicæ transplants, being evidently less developed. The fact that they were usually larger than the mucous glands,

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FIGS. 1 TO 6. *BI*, adjacent integument of back; *CE*, columnar epithelial cells; *DG*, dorso-lateral dermal plicæ skin graft transplanted to belly; *DI*, integument of dorso-lateral trunk region; *DP*, normal dorso-lateral dermal plicæ; *DPG*, dorso-lateral dermal plicæ developed in skin transplanted to the belly; *E*, thickened epidermis of dermal plicæ; *G*, granules of poison gland; *MG*, mucous gland; *P*, pigment masses; *PG*, poison gland; *T*, thickened stratum spongiosum of dermal plicæ; *VG*, belly skin graft transplanted to dorso-lateral dermal plicæ region; *VI*, integument of the belly.

FIG. 1. Histological section through dermal plicæ developed in skin graft from dorso-lateral dermal plicæ region previously transplanted to the back. Transplant sectioned three weeks following close of larval metamorphosis. × 112.

FIG. 2. Histological section through back skin graft previously transplanted to dorso-lateral dermal plicæ region. Transplant sectioned three weeks following close of larval metamorphosis. × 224.

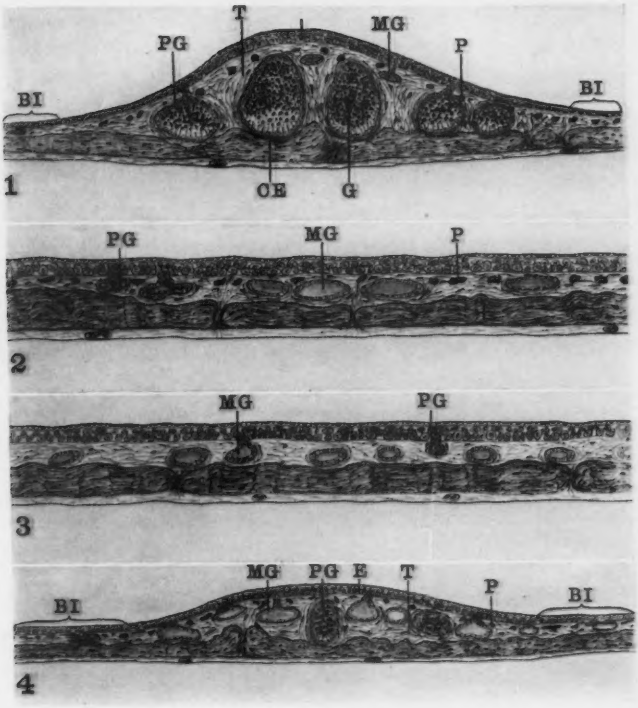
FIG. 3. Histological section through belly skin graft previously transplanted to dorso-lateral dermal plicæ region. Transplant sectioned three weeks following close of larval metamorphosis. × 336.

FIG. 4. Histological section through dermal plicæ developed in skin graft from lateral (jaw) dermal plicæ region previously transplanted to the back. Transplant sectioned three weeks following close of larval metamorphosis. × 112.

FIG. 5. Macroscopic appearance of skin graft from dorso-lateral dermal plicæ region, previously transplanted to the belly, three weeks following the close of larval metamorphosis. × 10.

FIG. 6. Macroscopic appearance of belly skin graft, previously transplanted to dorso-lateral dermal plicæ region, three weeks following the close of larval metamorphosis. × 10.

PLATE I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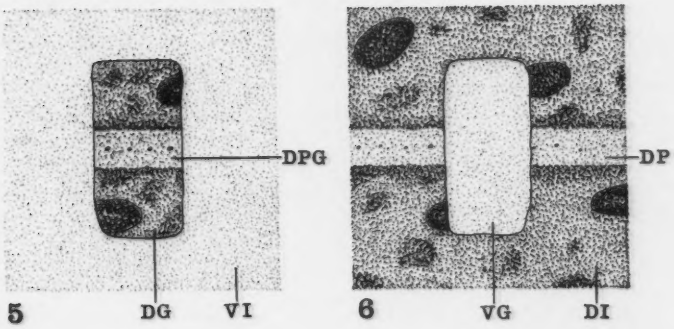
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however, probably accounted for the thickening of the stratum spongiosum present. The slight thickening of the epidermis and the moderate increase in thickness of the stratum spongiosum resulted in a convexity of the epidermal surface of the integument which, however, was not nearly as pronounced as in the case of the dorso-lateral dermal plicæ transplants. In general, it can be said that the histological appearance of the lateral dermal plicæ transplants on the back corresponded very closely to the microscopic picture of the normal lateral dermal plicæ of the same animal at this time.

Discussion

The results of the present work confirm those of Massie (1894) for the frog tadpole and of Wilder (1925) for *Eurycea* larvæ in that no anlagen of the acinous glands appear until just prior to the onset of metamorphosis. Likewise, the evidence is strongly indicative that they are derived from the Malpighian layer of the epidermis, separate developments being necessary for the two types of glands. There is no indication in the histological sections that transformation ever occurs from mucous to poison gland. In fact, as regards the dorso-lateral dermal plicæ regions especially, there is every reason to believe that the poison glands develop first and contain typical granular inclusions in the lumen at a time when the mucous glands are still in the anlage stage.

Study of the dermal plicæ in various stages of development leads to the conclusion that the progressive whitening of dermal plicæ integument is due to several factors. Of these the gradual reduction in the number of melanophores both in the epidermis and stratum spongiosum is probably of primary importance. This reduction in pigmentation apparently more than counterbalances the effects of increased thickening of the epidermis and stratum spongiosum. The latter layer, it may be stated, is apparently fully as dense in dermal plicæ regions as it is elsewhere throughout the integument. The extraordinary large size of the poison glands in dermal plicæ regions may also help to give the integument a certain degree of transparency and so facilitate the development of the characteristic white coloration of the plicæ as viewed externally.

The results of reciprocal, dermal plicæ skin transplantations with integument of the back and belly are especially clear in that certain narrow bands of larval integument are evidently physiologically specific for the development of mucous and large poison glands during larval involution. This specificity would also seem to include the ability to reduce pigmentation and to thicken the epidermis and stratum spongiosum. Apparently, therefore, neither the adjacent musculature or other local factors have any influence on the integument in the development of

dermal plicæ structures. This is borne out by the fact that back and belly skin transplanted to dermal plicæ regions failed to develop the slightest tendency towards dermal plicæ characteristics and that integument from dermal plicæ regions was capable of forming typical dermal plicæ structures at the usual metamorphic stage when transplanted to foreign locations. Logically, this specificity must be determined at some stage in the development of larval integument, the probability being that it is completed at a relatively early growth period of the larva, possibly at the same time that the integument of the embryo is formed. It would be interesting, in this connection, to make suitable integumentary transplantations on newly hatched tadpoles.

Granting that certain bands of integument are definitely specific for dermal plicæ formation during larval involution, the question naturally arises regarding the possible causative influences which evoke this differentiation and growth. It would seem probable that these influences are hormonic in nature and hence transported through the blood stream when released at a definite stage of metamorphosis. Possibly only an initial hormonic stimulus is necessary to bring about development of dermal plicæ structure or it may be that continued stimulation of this nature is essential for complete differentiation. Suitable homoplastic transplantations of integument, including half-formed dermal plicæ, to non-metamorphosing larvæ would undoubtedly answer this question.

The apparently direct influence of metamorphic hormones through the blood stream has been shown to effect the differentiation and growth or degeneration of other larval structures during anuran metamorphosis. The transformations so effected include the differentiation and growth of the fore-limbs (Helff, 1926), the hind-limbs (Schubert, 1926), the tongue (Helff, 1929*a*), the columella (Helff, 1929*b*), the nictitating membrane (Lindeman, 1929*a*); the histolysis of tail muscle (Helff and Clausen, 1929, and Clausen, 1929), and of tail integument (Lindeman, 1929*b*, and Clausen, 1929); and the development of the adult pigmentation pattern (Lindeman, 1929*b*). Preliminary work by the writer also indicates that the shedding of larval teeth during metamorphosis falls under the same category. Contrasted to this direct mode of hormonic influence, indirect mechanisms, involving first the degeneration or differentiation of other structures which in themselves then bring about other degenerations and differentiations, have been shown to apply to anuran metamorphosis. The transformations so initiated include the histolysis of opercular integument in the formation of the fore-leg perforations (Helff, 1926); the differentiation of the integumentary portion of the tympanic membrane (Helff, 1928); and the differentiation of

the yellow fibrous region of tympanic membrane lamina propria (Helff, 1929*b*).

Summary and Conclusions

1. The origin, time and separate development of the mucous and poison glands of *Rana pipiens* have been determined as they are found, especially, in dermal plicæ regions. The results confirm those of the majority of workers on other anurans and on urodeles.

2. Larval skin transplantations designed to test the specificity of various integuments for plicæ formation were made. These included autoplastic, reciprocal transplantation of skin known to normally develop dorso-lateral or lateral dermal plicæ with integumentary grafts from the belly and back. In such cases no demonstrable histological differentiation of glandular structures was observable at the time of transplantation. During metamorphosis, the various skin grafts developed the typical macroscopic and microscopic pictures of dermal plicæ characteristic of the region from which transplantation was made. Back and belly integumentary grafts transplanted to dermal plicæ regions maintained their specific histological characteristics, failing in every case to develop dermal plicæ structure.

3. The results are discussed and the conclusion reached that the potentiality to develop dermal plicæ and their histological components is determined and fixed in certain bands of integument at a very early stage of larval growth. The differentiation of this integument to form dermal plicæ structure is probably the result of direct hormonic influence through the blood stream at a definite stage of larval involution.

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ON THE CONDUCTION OF THE CORTICAL CHANGE AT FERTILIZATION IN THE STARFISH EGG

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A number of radical changes take place in the echinoderm egg when it is fertilized, one of which is a change in the cortex which prevents the entrance of a second sperm. That the egg is not entirely passive at the time of the entrance of the sperm is well known in a number of eggs from the phenomenon of the entrance cone which flows out and partly engulfs the sperm head.

For many years there has been a difference of opinion as to whether the fertilization membrane pre-exists in the sea urchin egg before fertilization. The more recent general opinion has been that some sort of a membrane is present. A. R. Moore (1929), however, has recently presented evidence which he regards as supporting Loeb's view that this membrane does not pre-exist but is formed *de novo* at the time of fertilization. In the case of the starfish egg, however, there is no question that a definite tough membrane pre-exists which normally lifts off following fertilization. Chambers (1921) has lifted this membrane from the unfertilized *Asterias* egg with a micro-needle. It has also been shown (Whitaker, 1928) that the cytoplasm of the egg of the starfish *Patiria miniata* may be readily divided into two separate parts with a micro-needle without severing the tough outermost membrane. If an egg which is divided in this way is inseminated, both fragments can be fertilized and will develop independently within the common fertilization membrane. In this case there can be no doubt that a morphological membrane is present, and that it lifts off as the fertilization membrane, even though the properties of the membrane may perhaps change following fertilization.

As early as 1878, O. Hertwig expressed the view that it is the egg plasma itself rather than the fertilization membrane which can prevent the entrance of a second sperm. Just (1919) has pointed out that obviously the fertilization membrane rises off the egg so late after the contact of the fertilizing sperm that another and more rapid change of the cortex must be postulated to explain rejection of the second sperm.[1]

[1] In *Arbacia* the membrane begins to lift off about eighteen seconds after contact of the fertilizing sperm, and in *Echinarachnius* after about twenty to forty seconds (from Just, 1929).

Just has further observed in *Echinarachnius* that this change, which he calls a " wave of negativity," spreads rapidly from the point of contact of the first sperm, just as the lifting of the fertilization membrane itself spreads from this point, but spreading much more rapidly so that it has covered the entire egg before the fertilization membrane has even started to rise at the entrance point of the sperm. His evidence for this " wave of negativity " is based on observations on living eggs. As soon as the tip of the first sperm has penetrated the egg, other sperm are not engulfed in the immediate vicinity of the first sperm, although they may be taken in further around the egg. As the first sperm penetrates still further, the " wave of negativity " progresses further around the egg until at the moment the sperm head has entirely disappeared into the egg, only the opposite pole of the egg can engulf another sperm. Sperm which become attached but do not enter the egg are lifted off as the fertilization membrane rises.

More recently Just (1929) and others have observed that in eggs which are in excellent condition, the cortical reaction is so rapid as to be practically instantaneous. It is practically impossible to produce polyspermy by the use of concentrated sperm suspensions. It is evident that in such innoculations the time elapsing between the contact of the first and the contact of the second sperm must be an exceedingly small fraction of a second. The spread of the cortical change in eggs in perfect condition is much too rapid to be traced by the eye. Just (1930, page 337) says, " At least for the normally monospermic ova of the marine forms which I have studied, it is certainly true that if they are in optimum condition, polyspermy is difficult if not impossible. Such ova in order to become polyspermic must undergo treatment which impairs their cortices. . . . Doubtless the very instant that contact between spermatozoon and ova is made, polyspermy is blocked."

The response of the egg to the stimulus of the first sperm involves the whole interior of the egg as well as the cortex. Flowing of the interior protoplasm immediately succeeds fertilization, as well as changes in viscosity, etc. (Heilbrunn, 1928). The interior changes may be secondary, however,—that is, consequences of changes in permeability and surface tension of the cortical region.

That the cortex performs a necessary function in admitting the first sperm, as well as in preventing polyspermy, has been shown by Just (1923) for *Echinarachnius* and for *Arbacia* and by Chambers (1921) for the starfish *Asterias*. Just burst eggs by returning them from hypertonic to normal sea water, and also by passing eggs through fine-meshed bolting silk and through lens paper to obtain endoplasmic buds or fragments bearing none of the original cortex of the egg. Chambers

obtained similar fragments by means of the microdissection needle. In none of these eggs did the endoplasmic buds react to sperm or become fertilized.

The present observations add little evidence as to the nature of the cortical reaction. They are concerned with the locus or site of conduction of the spreading impulse. If it starts at a point on the cortex, it must spread either in the form of the cortical change itself, or else it must be an equilibrium shift transmitted either around the cortex, or through the egg, giving rise to the cortical change.

In a previous paper on the development of fragments of *Patiria* eggs (Whitaker, 1928), 69 cases are recorded in which two completely separated fragments lying within the same fertilization membrane-to-be were inseminated and became fertilized independently, one sperm entering each fragment. This result is almost invariably obtained when the two masses of cytoplasm are not in contact at the time of insemination. This seems to show that the impulse which spreads through the egg and results in the cortical change which prevents the entrance of a second sperm does not pass through this outermost membrane. There is no evidence of injury to the outer membrane as a result of pinching the protoplasm. It lifts off in the normal way after fertilization, although sometimes it is temporarily constricted where the needle has pressed. Additional experiments have been made in which eggs have been pinched into two cytoplasmic fragments lying within a common outer membrane, and the fragments have been allowed to flow together before insemination. In this case the treatment of the outer membrane is practically identical with that in the cases of fragments which remain apart, the difference between the two types of cases being only in the fusion or non-fusion of the protoplasm within the membrane. Only sets of eggs were used in which control tests gave 98–100 per cent fertilization. A moderately concentrated suspension of sperm was used. As a control, to test for polyspermy, five or six normal eggs were placed by means of a mouth pipette around the experimental egg, not more than several egg diameters away. Half an hour after insemination the experimental egg was transferred to a separate dish, since at the time of cleavage when the eggs are changing shape there is otherwise some danger of mistaken identity. For some time after insemination, however, the marks of cutting are clearly discernible on the experimental egg. In all cases the eggs were left in fresh sea water for about ten minutes after cutting before insemination. It has been found in a number of echinoderm eggs that this procedure permits the fragments to recover from the operation and in the case of *Arbacia* eggs, for example, it avoids polyspermy which often results from immediate insemination.

The degree of separation of two cytoplasmic fragments lying within the same outer membrane can be regulated by the extent to which the membrane is stretched with the needle, a very slight difference in the amount of stretching determining whether the fragments will flow back together or not. The eggs of *Patiria* are large, averaging about 190 or 200 microns in diameter, and they are comparatively clear, so that the grosser aspects of the nuclear and astral phenomena can usually be clearly seen in the living egg or egg fragment.

Eleven eggs were cut, after the fashion of the 69 cases already quoted, so that the two fragments were separate at the time of insemination. The control eggs lying near the experimental egg showed no polyspermy. In 9 of the 11 cases, both fragments were fertilized independently, each dividing with a single amphiaster into two normal cells (Fig. 1, *a, b, c*). In two cases one fragment only became fertilized with one sperm, the other fragment failing to become fertilized.

Twenty-five eggs were cut into two fragments and allowed to flow completely together before insemination. In 18 of these cases the fragments remained separate for one to two seconds, in three cases for more than five seconds, and in four cases for fifteen seconds to one minute. If the fragments remain apart for several minutes, a solidification of the cut surface usually prevents a complete refusion of the fragments, or in cases of fusion results in an ectoplasmic wall along the surface of fusion. In the 25 cases of complete fusion, 23 cleaved with one amphiaster into a normal two-cell stage. Apparently only one sperm had entered. There were no cases of polyspermy in the control eggs. In two cases division was by means of a triaster into three cells, apparently due to the entrance of two sperm. One of these cases of polyspermy was an egg separated for one to two seconds. The controls showed no polyspermy. The other was an egg which had been apart for more than fifteen seconds. The controls in this last case showed polyspermy, probably indicating that insemination was too heavy and that the eggs were not in the best condition.

The results show that when a sperm enters one part of an egg, usually no other sperm enters any other part of the egg which is joined by protoplasmic fusion, but another sperm does enter a part of the egg which has only the same outer membrane in common, equally well as if the parts of the egg were entirely separated and removed from one another. Apparently the cortical change which normally prevents polyspermy is not conducted through the outermost membrane, the fertilization membrane-to-be. It is conducted through the protoplasm within this membrane.

The question naturally arises: To what extent must two fragments

be associated in order that the fertilization of one shall prevent the fertilization of the other? Is mere contact of the surfaces of two fragments within the outer membrane sufficient or is fusion necessary? In contrast with our extreme cases of complete separation and complete fusion, which can be identified with certainty and in which the results are clear cut, it is difficult to tell by observation in the border line cases in which the two fragments are in contact precisely what degree of fusion has taken place. Fragments which are in contact but not fused at the time of insemination usually fuse soon after fertilization. Although no statement can be made at present as to the minimum degree of contact or fusion necessary to transmit the impulse, the following experiments throw some light on the question.

Fifteen eggs were cut into two fragments which remained separate for more than a minute and then came together so that at the time of insemination they are classed as (1) touching, (2) in good contact, or (3) fused at a narrow neck, the neck being from less than ten to thirty microns through. The eggs are 200 microns in diameter. In five cases the fragments were touching. In four of these cases one sperm entered each fragment. The fragments subsequently fused, but retained an ectoplasmic partition and separated at the time of cleavage, each com-

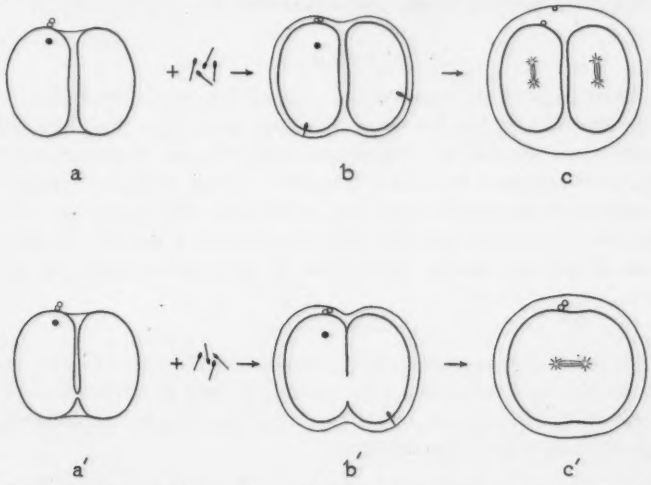

FIG. 1. Diagrams before insemination, after insemination, and just before cleavage, of eggs cut without severing the outermost membrane. (Sperm disproportionately enlarged.)

a, *b*, *c*. An egg with halves separated at time of insemination.

a′, *b′*, *c′*. An egg with halves joined by a narrow neck at time of insemination.

ponent dividing with a single amphiaster into two cells. The fifth case admitted only one sperm and divided as a whole into two cells. In three cases the fragments were in good contact, that is, about on the border between touching and slightly fused. Two of these cases had one amphiaster and divided into two cells. The third admitted a sperm into each half and divided directly into four cells. In seven cases the two halves were truly fused by a narrow neck at one end of the cut. Shortly after fertilization more complete fusion took place. In all of these seven cases only one sperm entered the joined fragments. In four of the cases normal division into two cells followed (Fig. 1, *a'*, *b'*, *c'*). In two cases the nucleus of the sperm, which had entered the non-nucleated fragment of the egg, was not able to pass through the gelated partition of the fused surfaces to fuse with the egg nucleus, and only the fragment containing the sperm nucleus divided into a normal two-cell stage. The other half separated off at the time of division but did not itself divide. In the seventh of these cases the sperm entered the non-nucleated fragment, which divided, and the nucleated fragment which apparently received no sperm divided late, involving the egg nucleus only.

These results on fragments which are only in slight contact show that a very narrow neck of truly fused protoplasm conducts the impulse. Mere contact without fusion probably does not.

Summary

When eggs of the starfish are pinched into two cytoplasmic fragments which lie within the same outermost membrane (the fertilization membrane-to-be), and are inseminated while the two fragments are separate, each fragment receives one sperm. If the fragments completely fuse before insemination, only one sperm enters the fused egg. If the fragments are merely touching, each may receive a sperm. If they are joined by a very narrow fused neck of protoplasm, only one sperm enters.

Conclusion

The outermost membrane of the starfish egg (the fertilization membrane-to-be) does not conduct the cortical change at fertilization which prevents the entrance of a second sperm. This change is conducted by the protoplasm within the membrane.

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A STUDY OF THE EFFECTS OF MODIFICATIONS OF THE CULTURE MEDIUM UPON LENGTH OF LIFE AND FECUNDITY IN A ROTIFER, *PROALES SORDIDA*, WITH SPECIAL RELATION TO THEIR HERITABILITY

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Introduction

Extensive investigations on the Infusoria have demonstrated that these organisms, while undergoing continuous vegetative reproduction, under certain conditions " run down,"—become depressed. In many of the earlier genetic studies this progressive depression was interpreted to mean that the organisms have a definite life cycle with periods of youth, maturity, and old age, representing through the course of many consecutive generations of vegetative reproduction the usual life cycle of a single metazoan organism. The results of later investigators, however, show that in many cases the depression is partly or entirely due to unfavorable environmental conditions. Enriques, Woodruff, and others found that under adequate cultural conditions certain Infusoria can be cultivated indefinitely without decline in vitality, although the same Infusoria under poor cultural conditions exhibit a depression which may continue for long periods, becoming more and more marked as generations pass and eventually resulting in the death of most of the lines.

The inheritance of such a depression, after restoration to normal conditions, was tested by Middleton (1918) and by Jollos (1921). Middleton found that a depressed condition induced in *Stylonchia pustulata* by long continued culture at a high temperature is inherited for at least twenty days after restoration to normal temperature. Jollos produced a depression of the fission rate in *Paramecium aurelia* by subjection to calcium nitrate; he found that this lowered rate of reproduction is heritable for four months. In most other cases it has not been possible to test critically the inheritance of the depression after restoration to normal conditions, but it is commonly assumed that inheritance occurs. (For a detailed review of this type of work on the Protozoa, see Jennings, 1929.)

The present investigation was undertaken to determine how far

conditions in the lower Metazoa are comparable to those in the Infusoria, particularly as to the production of depression, its increase as generations pass, and its inheritance after return to normal conditions. The Rotifera are particularly suitable for such an investigation. They resemble the Infusoria in size, habitat, and mode of life, but reproduce by means of germ cells which undergo a process of development in the production of the many-celled individual. They can be subjected to the same conditions that induce depression in the Infusoria, and the depression so produced can be studied in relation to its tendency to cumulation and its persistence after return to normal conditions.

The particular species of rotifer chosen, *Proales sordida* Gosse, has the additional advantage of reproducing exclusively by parthenogenesis, that is, from single parents, as do the Protozoa in vegetative reproduction. The complications of biparental inheritance, common to most types of germ-cell reproduction, are therefore avoided.

Insufficient feeding was the method used for producing depression: certain lines were well-fed; other lines were given insufficient food in definite ratios to the amount given the well-fed group. This method is the one used by Beers (1926) with the infusorian *Didinium*. He found that his well-fed group exhibited a high fission rate throughout his experiment; and that his inadequately-fed group showed from the first a lower fission rate, which after some time became still further depressed, this depression steadily increasing until the line encysted.

Previous experimental work on the Rotifera has been concerned chiefly with the problem of sex-determination; but there are a few papers which bear on the questions here considered. Among these are four on the effects of food control during parthenogenetic reproduction. Luntz (1926), in a critical study of the relation of food to the onset of the sexual generation in *Pterodina elliptica*, describes briefly an experiment on quantitative variation of food. Using Beneke's solution as the culture medium, and *Polytoma* or *Chlamydomonas* as the food, he varied the amount of available food by varying the number of drops used. He found that the number of offspring produced varied directly and precisely with the amount of food provided. He does not give data for successive generations; this work therefore furnishes no data on cumulative effects.

Shull (1912) and Whitney (1912*a*) carried on extensive investigations bearing upon this matter, in successive generations of the rotifer *Hydatina senta*. Both of these investigators, using various types of culture media, found that races reproducing exclusively by parthenogenesis decline in vitality, as indicated by their lowered fecundity. Whitney suggests that this may be due to "the constant environment

of the horse manure cultures" (1912*a*, p. 343). Lambert, Rice, and Walker (1923), working under the direction of Whitney, show that this decline in vitality is due to the unbalanced diet, not to the parthenogenetic method of reproduction. None of these four investigations take up the question of the heritability of these depressions.

Other investigations, in which alcohol is used as the modifying factor, do deal with the heritability of modifications produced. Whitney (1912*c*) subjected *Hydatina senta* to alcohol and produced a decrease in fecundity which continued for two generations after return to a normal culture medium, but not longer. Noyes (1922) with *Proales decipiens,* and Finesinger (1926) with *Distyla inermis,* produced a cumulative decline in fecundity by subjection to alcohol and found that on return to normal conditions the depression continued to some degree for two generations only. (Finesinger, however, found that in weak doses alcohol acts as a stimulant and causes a slight increase in both fecundity and longevity.)

In the present study, dealing with the effects of a reduced food supply, two main questions are considered: First, do adverse environmental conditions produce the same effects in the Rotifera as in the Infusoria; that is, do they bring about a decline which increases with succeeding generations, so that the Rotifera run down as do the Infusoria? Second, if they become depressed, is this depression inherited in succeeding generations when the progeny are returned to a favorable environment?

Two characteristics, fecundity and length of life, were taken as indices of vigor. The effect of the reduced food supply on these indices was studied during the summer of 1928; the results of these experiments are presented in Part I. During the summer of 1929 the experiments, somewhat modified, were repeated; their results are presented in Part II. All of the experimental work was done in the Marine Biological Laboratory at Woods Hole.

The investigation was undertaken at the suggestion of Professor H. S. Jennings. It gives us great pleasure to express our appreciation of his advice throughout the investigation and his help in the preparation of the manuscript.

The Organism

Proales sordida is a rotifer well suited to this type of investigation. It lives in ponds and sluggish streams, and can be cultivated with ease in the laboratory in almost any infusion favorable to the growth of Protozoa; it feeds on bacteria and other minute organisms. The usual length of life is eight days, though occasionally individuals live as long as twenty-five days. Each animal produces twenty-four to twenty-

eight eggs, with an observed maximum of thirty-four eggs. The adults average 300 microns in length. Reproduction is exclusively parthenogenetic. The species consists entirely of females; no males have ever been described and none have appeared during the history of our cultures even under conditions known to be favorable to male production in other rotifers. It was therefore possible, by starting with a single ancestor, to have all the animals used in these experiments members of a single clone, forming a group as genetically similar as it is possible to obtain. (For a more detailed description of this organism see Jennings and Lynch, 1928, I.)

Methods

It is not only necessary, for this type of experimentation, to have animals that are alike genetically; it is also necessary to have them as uniform as possible in other respects. Uniformity in age is particularly important, since Jennings and Lynch (1928, I) have demonstrated that the offspring of young parents have a lower fecundity than the offspring of the same parents when old. *Proales sordida* has an average life of only eight days; it begins to lay eggs about forty-eight hours after hatching. Its eggs, laid in the one-cell stage, take from twenty to twenty-four hours to hatch. A population uniform in regard to age may therefore be obtained by including in it only such eggs as are laid within a five-hour period by parents differing in age by less than twenty-four hours. To meet these conditions the following procedure was employed: In starting each experiment, several thousand eggs were removed from mass cultures and placed in oatmeal infusion in culture dishes, three hundred to a dish. Twenty-four hours later a small amount of infusion was added to each dish; at that time most of the eggs had hatched. After the lapse of another twenty-four hours, when most of the animals had begun to lay eggs, the animals were transferred to dishes of fresh culture medium. The eggs laid within the next five hours were removed and placed on two-concavity slides, one egg in each concavity in a single drop of culture medium. The animals hatching from these eggs were the first generation of experimental animals. By this procedure, eggs of approximately the same age were obtained from parents of about the same age, thus fulfilling the requirements outlined above. Throughout the remainder of the investigation, except in rare cases to be mentioned at the appropriate places, no eggs later than the third were used to give rise to a new generation.

Without exception the animals were cultivated individually on ground-glass, two-concavity slides in single drops of culture medium. The slides were kept on glass supports in nine-inch crystallizing dishes, twelve slides to a dish. Each dish was water-sealed by placing boiled

spring water in the bottom of the larger portion and inverting over it the smaller portion.

The animals were examined at approximately twelve-hour intervals. In the morning each animal was transferred by means of a capillary pipette to a fresh drop of culture medium on a clean slide. In the evening the eggs which had been laid during the day were removed from the drops, but the animals were left undisturbed. The capillary pipettes were sterilized in boiling water before each transfer. Pipettes of the same size were used to place the culture fluid on the slides. The pipettes were boiled daily in distilled water and the rubber bulbs were boiled at frequent intervals.

A favorable culture medium for *Proales* is an oat infusion prepared by boiling fifteen flattened flakes (¼ gram) of rolled oats ("Quaker Oats") for three minutes in 100 cc. of spring water, filtering this, and allowing it to stand for twenty-four hours before using (*cf.* Jennings and Lynch, 1928, I). The strength of this culture fluid may be easily varied by changing the number of flakes of oatmeal used. Such varied solutions were used in these experiments. In every case the media were prepared simultaneously in the same way, the only difference between them being the number of flakes of oatmeal used.

Part I. Experiments of 1928

In this series of experiments the following strengths of culture medium were used:

Control Series	*C*, full-strength	fluid,	15	flakes	oatmeal	to	100	cc.	spring	water
Test "	*H*, half-strength	"	8	"	"	"	"	"	"	"
" "	*Q*, quarter-strength	"	4	"	"	"	"	"	"	"
" "	*S*, boiled spring water only									

These media were prepared as described above and were left exposed to the air for twelve hours before being corked.

In the preliminary experiments it was found that the animals would not live beyond the second generation in boiled spring water without oatmeal (*S* culture medium), nor in the four-flake solution (*Q* culture medium).

In the main experiment, begun July 19, 1928, and continued for three months, three groups of animals were studied: one group (*C*) was cultivated in full-strength culture medium; a second (*H*) in half-strength culture medium; and a third (*Q*) was put for its first generation into quarter-strength culture medium, into full-strength for its second generation, and then again into quarter-strength culture medium, where it remained until its death. Some individuals of the later genera-

tions of both the *H* and the *Q* groups, and their descendants, were cultivated in full strength medium (*HC* and *QC* groups).

At the start, one hundred individuals in each generation of all three groups were kept throughout their lives. But the rapid increase in coexisting generations so increased the number of animals to be examined daily that a change in procedure was necessary. In most cases the number of individuals to a generation was reduced, usually to about fifty (*cf.* Tables I and II). In other cases, not only were the members of a given generation reduced in number, but they were kept just long enough to produce the next generation, and then discarded. Such generations, of course, yielded no data on fecundity or longevity.

TABLE I

Experiment I, 1928—Showing for the successive generations (Gen.) of the two control series *C* and *M* the total numbers of eggs isolated (No.); the numbers of these that did not hatch (*N.H.*), and the numbers that hatched (*H.*); and for the latter the mean numbers of eggs laid and the mean numbers of days lived. Data presented graphically in Figs. 1 and 2.

C Series (full-strength medium)						M Series (full-strength medium)					
Gen.	No.	N. H.	H.	Mean Eggs	Mean Days	Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	92	1	91	7.67	5.76	1	49	1	48	3.60	5.23
2	106	5	101	6.02	4.85	2	95	7	88	1.93	4.32
3	47	6	41	6.41	5.02	3	51	16	35	7.88	7.02
4	52	7	45	6.22	4.77	4	50	2	48	11.31	9.56
5	57	3	54	6.18	4.49						
6	58	8	50	3.02	3.74	9	49	1	48	9.31	11.61
7	49	8	41	1.95	3.68						
8	52	3	49	2.55	4.11	11	49	0	49	8.85	9.45
9	75	17	58	1.77	4.00						
10	56	6	50	2.74	4.18	13	42	1	41	11.58	9.17
11	51	7	44	2.40	4.38						
12	60	12	48	2.04	3.51						
13	49	4	45	1.73	3.18						
14	48	3	45	1.66	3.75						
15	49	5	44	1.36	4.15						
16	40	7	33	1.81	4.59						
17	40	4	36	2.16	4.56						
18	45	6	39	5.41	6.25						
19	49	4	45	12.17	10.06						
24	51	1	50	8.86	10.70						
26	39	1	38	12.57	10.71						
28	44	0	44	16.52	11.44						

TABLE II

Experiment I, 1928. Showing for the successive generations (Gen.) of the *H* series (cultivated in half-strength medium), the *Q* series (cultivated in quarter-strength medium), and the *HC* and *QC* series (cultivated in full-strength medium), the total numbers of eggs isolated (No.), the numbers of these that did not hatch (*N.H.*), and the numbers that hatched (*H.*); and for the latter the mean numbers of eggs laid and the mean numbers of days lived.

H Series (half-strength medium)

Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	97	1	96	2.16	3.69
2	93	0	93	2.19	3.69
3	87	6	81	2.54	3.16
4	52	10	42	3.97	3.78
5	46	2	44	3.43	3.95
6	62	0	62	2.41	3.64
7	45	3	42	2.11	3.42
8	45	4	41	1.24	2.85
9	43	6	37	0.94	2.75
10	18	2	16	0.62	2.50
*11	7	0	7	0.14	3.00
*12	10	0	10	0.50	2.25
*13	4	1	3	0.00	1.83

HC Series (full-strength medium)

*1(10)	14	2	12	2.50	3.95
*2(11)	27	4	23	1.04	3.19
*3(12)	23	7	16	1.62	2.90
*4(13)	24	3	21	2.90	4.57
5(14)	48	1	47	1.91	4.25
6(15)	58	10	48	2.14	4.37
7(16)	46	6	40	2.47	4.72
8(17)	45	5	40	6.45	6.83
9(18)	49	7	42	9.90	8.61
14(23)	41	2	39	8.07	11.14
16(25)	39	0	39	10.38	10.23
18(27)	49	0	49	15.14	11.15

Q Series (quarter-strength medium)

Gen.	No.	N. H.	H.	Mean Eggs	Mean Days
1	100	0	100	0.18	2.01
†(2)	17	0	17	7.76	5.50
3	55	8	47	0.95	1.96
*4	17	0	17	2.00	3.52
5	34	2	32	1.68	3.09
6	49	1	48	1.02	2.70
7	49	0	49	0.48	2.53
*8	13	1	12	0.00	1.75

QC Series (full-strength medium)

*1(8)	11	2	9	4.00	4.72
*2(9)	33	6	27	1.29	3.77
3(10)	34	4	30	1.30	3.30
*4(11)	35	13	22	0.90	2.95
*5(12)	20	3	17	0.64	3.26
*6(13)	9	0	9	0.77	3.88
*7(14)	3	0	3	2.00	4.33
*8(15)	2	0	2	5.50	7.75
*9(16)	8	0	8	8.12	9.18
10(17)	44	4	40	5.50	7.80
11(18)	46	3	43	6.06	8.38
12(19)	47	0	47	5.48	8.70
13(20)	54	0	54	6.98	10.24
15(22)	47	0	47	9.68	9.95
17(24)	50	0	50	14.34	11.01

†(2) Grown in full-strength medium.
* Generations comprising less than thirty animals.

General Description

I. The Control Series.

During the course of the experiment the control animals lived through twenty-eight generations, showing great variability in length of life and fecundity. This variation appeared to be correlated with the temperature fluctuations. The temperature was high when the experiment began and remained high for sixteen generations; these generations showed a progressive decline in both fecundity and longevity. The succeeding generations (17th to 27th), subjected to a lower temperature, showed a recovery which started with the first generation grown at the cooler temperature. This recovery was so swift, both fecundity and longevity increased so rapidly, that with the 20th generation it became necessary to reduce the number of animals examined. This was done by testing only occasional generations, as noted above. The number of animals in the generations not tested (generations 20, 21, 22, 23, 25, and 27) was re-

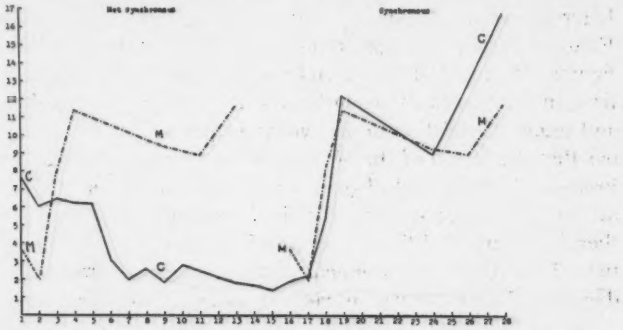

FIG. 1. Experiment I, 1928. Comparison of the two control series *M* and *C*, based on egg production; horizontal axis represents successive generations; vertical axis, mean egg production.

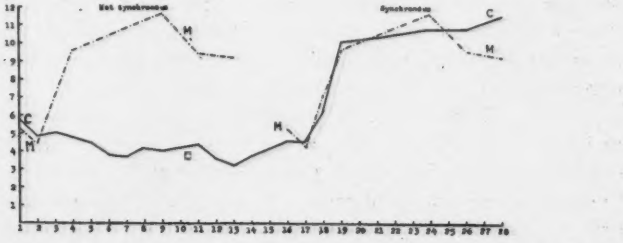

FIG. 2. Experiment I, 1928. Comparison of the two control series *M* and *C*, based on length of life; horizontal axis represents successive generations; vertical axis, mean length of life in days.

duced to thirty, and these were maintained only long enough to provide the next generation.

When the control (*C*) animals were in their most depressed condition (generation 15) a special effort was made to determine the cause of their depression by comparing them with a second series of controls, taken at this time from mass culture. This new control series (termed *M*) was treated in every way like the original control series, so that the only known difference between the two sets was the difference of date of derivation from mass culture, resulting, of course, in a diversity in duration of cultivation on slides. If duration of slide cultivation is the effective cause of depression, the early generations of *M* should not show depression; there should be a wide diversity between their records and the records of the synchronous generations of *C* (16th to 28th generations undergoing slide cultivation). If, on the other hand, some current environmental factor is the effective cause, synchronous generations of the two series should yield like data. Figures 1 and 2 demonstrate the latter condition. *C* and *M* show a similar and synchronous modality. Figure 1 is based on egg production; Fig. 2 on length of life. In both figures the record of the second control series *M* is shown in two ways; first, in relation to the records of the early generations of the *C* series, and second, in relation to the synchronous generations of *C*. It is obvious that the record of the *M* series, comprising thirteen generations, follows closely the record of the generations of the *C* series that were living at the same time; not the record of the early generations of *C*. It is therefore concluded that the cause of the depression in *C* was some feature of the current environment, probably the high temperature; and that duration of slide cultivation had had no depressive effect.

II. The *H* Series.

The series cultivated in half-strength culture medium (*H*) was consistently lower in fecundity and length of life than the controls. Consecutive generations showed some variation, but became progressively depressed, and the series died out in the 13th generation. In Table II are given the records of series *H* for every generation, showing the mean egg production and the mean length of life.

Fortunately the ultimate extinction of the *H* series was anticipated and some eggs of the ninth generation were placed in the full-strength culture medium, thus beginning a group of animals (series *HC*) in which to test recovery from depression. At the end of the experiment the *HC* group had been cultivated continuously for eighteen generations in the full-strength culture medium. It showed complete recovery from the depression (see Table II, *HC*). Generations 19, 20, 21, 22, 24, and 26 were not tested.

III. The *Q* Series.

In the preliminary experiments the animals cultivated in the quarter-strength culture medium had not survived beyond the second generation. The exceedingly low records of the first generation of the *Q* series of the main experiment indicated that they were following a similar course. Consequently, it was thought best to rear the animals of the second generation in full-strength culture medium. The records of this second generation of *Q*, cultivated in the full-strength culture medium, greatly exceeded those of the second generation of *C* animals. But the records of the third generation, cultivated in the quarter-strength culture medium, were again very low, although not as low as those of the first generation. The subsequent generations were grown continuously in the quarter-strength culture medium; they declined consistently and died out in the eighth generation.

A few eggs from the seventh generation were placed in the full-strength culture medium to test the recovery of the *Q* series. This *QC* series was continued in the full-strength solution until the end of the experiment. They improved, but their records on the whole were lower than those of the controls (*C* series) and lower than those of the *HC* series. The generations did not overlap as much in this group as in the other two groups, and at the end of the experiment, when the *H–HC* series was in its 27th generation and the *C* series was in its 28th generation, the *Q–QC* series was only in its 24th generation. Generations 21 and 23 were the only ones not tested. The record for each generation of the *Q–QC* series is given in Table II. Because of the low fecundity of these depressed animals the number of individuals in many generations was small, although all the eggs laid were kept, including in some cases eggs later than the third.

Temperature Variation

During the summer of 1927 the laboratory in which these experiments were conducted had shown fluctuations of less than two degrees (*cf.* Jennings and Lynch, I, p. 369), and it was expected that it would maintain the same constancy during the summer of 1928. Unfortunately this was not the case. The temperature of the Woods Hole region was unusually variable from July until September of that year, and the laboratory showed correspondingly greater fluctuations.

The temperature was read at twelve-hour intervals from a Maximum-minimum thermometer which was suspended above the rotifer cultures. The data thus obtained are presented in Fig. 3, which is designed to show the relation between the temperature fluctuations and the

records of the control animals (*C* series). On the vertical axis the temperature is plotted in degrees centigrade; on the horizontal axis the time is plotted in twelve-hour periods, for the duration of the experiment. The upper, shaded curve (*a*) shows the maximum and minimum records for each twelve-hour period, the upper line showing the maximum, the lower line the minimum. The second curve (*b*) is a simplified form of

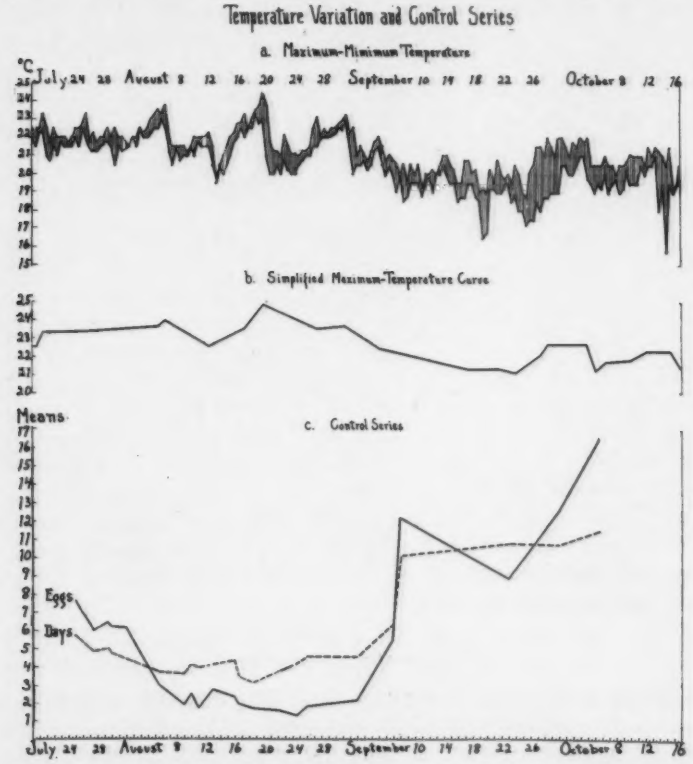
Temperature Variation and Control Series
a. Maximum-Minimum Temperature
°C
July 24 28 August 8 12 16 20 24 28 September 10 14 18 22 26 October 2 6 12 16
b. Simplified Maximum-Temperature Curve
Means
c. Control Series
Eggs
Days
July 24 28 August 8 12 16 20 24 28 September 10 14 18 22 26 October 2 6 12 16

FIG. 3. Experiment I, 1928. Chart showing temperature variation and the records of control series *C*. (*a*) Upper shaded curve: the maximum and minimum temperatures at twelve-hour intervals from July 19th to October 16th; the upper line shows the maximum temperatures; the lower line shows the minimum temperatures. (*b*) Middle curve: a simplified form of the temperature curve giving only the major points of maximum temperature. (*c*) Lower two curves: the record of control series *C* for egg production (solid line) and for length of life (dashed line). The median dates of the twenty-eight generations produced from July 25th to October 5th are indicated on the horizontal axis; the mean number of eggs produced and the mean number of days lived by each generation are indicated on the vertical axis.

the upper line of the shaded chart and shows the highest records of the summer plotted at intervals of approximately one week. The third curve (*c*) shows the records of the control series (*C*) plotted on the same basis as in the foregoing curves. On the vertical scale are plotted the mean number of days of life (dotted-line curve) and the mean number of eggs (solid-line curve) for each generation. On the horizontal scale is recorded the time, as in the upper curves. The records of the controls are plotted by generations on their "Median Dates"; that is, on a date exactly midway between the date when the first animal of a given generation was isolated as an egg and the date when the last animal of that generation died.

A comparison of the three curves (*a*), (*b*), and (*c*), clearly demonstrates a close relation between the records of the control animals and the temperature. As the temperature became higher the animals became more depressed, the lowest records of the controls for the entire experiment being made immediately after the highest temperature of the summer. The animals improved when the temperature became lower. At the end of the summer, when the temperature stayed consistently lower, they gradually recovered from their depression.

Analysis of the Data

I. Method.

Inasmuch as the temperature influenced the results in such a striking way, valid comparisons of the different groups of animals could be made only if the groups to be compared lived at the same time and were thus subjected to the same temperature variations. An arbitrary method was devised to meet this condition. This method was a day by day comparison made on a percentage basis with the control series *C* as the standard.[1] The daily average record of the control series was taken as 100 per cent. The averages for the other cultures on the same day were then expressed as percentages of this. Thus if the average number of eggs produced by *H* on a given day was one-half as great as the number produced by the controls, *H* was given a rating of 50 per cent. In calculating the averages for the controls, the generations as such were disregarded, since, in an adequate medium, the number of generations cultivated on slides had been shown to be unimportant (p. 38). In calculating the records of *H* and *Q*, cultivated in the reduced media, each generation was considered separately.

The procedure for determining the percentage for each generation of an experimental group was as follows, using *H* as an example: First, the mean number of eggs for animals of a given generation of *H* was

[1] This method was devised by Dr. Daniel Raffel of this laboratory.

computed by days on the basis of the day they were begun as individuals. Then this mean for *H* was expressed as a percentage of the mean of *C* for the same day. In this way percentages were calculated for each day

TABLE III

Experiment I, 1928—Showing for the successive generations (Gen.) of the four test series (*H, Q, HC, QC*) the numbers of animals (No.) and the percentages of fecundity and longevity, computed as described in the text (pp. 41–43). Data presented graphically in Figs. 4, 5, 6 and 7.

H Series (half-strength medium)				*Q* Series (quarter-strength medium)			
Gen.	No.	Fecundity	Longevity	Gen.	No.	Fecundity	Longevity
		per cent	*per cent*			*per cent*	*per cent*
1	96	28.16	64.06	1	100	2.34	34.89
2	93	38.22	75.78	†(2)	17	126.57	108.65
3	81	98.48	75.40	3	47	16.67	41.55
4	42	111.79	84.91	*4	17	40.98	82.81
5	44	85.69	94.54	5	32	70.47	82.68
6	62	80.67	92.01	6	48	55.61	71.99
7	42	115.65	95.60	7	49	35.85	62.92
8	41	69.01	70.67	*8	12	0.00	41.83
9	37	39.34	65.58				
*10	16	24.72	59.22				
*11	7	65.09	74.85				
*12	10	46.88	67.60				
*13	3	0.00	50.15				
HC Series (full-strength medium)				*QC* Series (full-strength medium)			
*1(10)	12	85.58	89.67	*1(8)	9	140.74	111.46
*2(11)	23	47.75	88.16	*2(9)	27	59.49	93.08
*3(12)	16	104.80	90.83	3(10)	30	83.79	100.47
*4(13)	21	190.96	118.52	*4(11)	22	51.21	78.81
5(14)	47	123.75	98.97	*5(12)	17	42.50	82.52
6(15)	48	115.89	94.68	*6(13)	9	46.99	87.35
7(16)	40	129.71	102.81	*7(14)	3	97.05	91.99
8(17)	40	114.86	110.12	*8(15)	2	261.62	155.04
9(18)	42	80.18	84.93	*9(16)	8	87.20	97.54
				10(17)			
14(23)	39	81.83	111.33	11(18)	No controls for comparison ‡		
				12(19)			
16(25)	39	83.14	95.00	13(20)	54	79.06	93.03
18(27)	49	91.47	97.30	15(22)	47	70.49	92.62
				17(24)	50	86.45	95.91

* Generations comprising less than 30 animals.

†(2) grown in full-strength medium.

‡ Gen. 17, 18, 19 of the *QC* series were begun at a time when no control animals were begun, so comparison on the percentage basis was impossible.

included in that *H* generation. Finally, the percentages were weighted according to the number of *H* animals included in each, and the true mean of these percentages was found for each generation. Following this method, the records of fecundity and longevity of each generation of the experimental groups were calculated as percentages of the synchronous records of the *C* group. The variation of these percentages with the passing of generations is shown graphically in Fig. 4; here the vertical axis represents the percentages, the horizontal axis represents the generations, and the straight horizontal line at 100 per cent represents the standard of comparison,—the record of the *C* group.

A dependable interpretation of the data must take into consideration the numbers of animals involved; unless this number is fairly large, the sample may not be representative. In this study, generations comprising less than thirty animals have been considered of doubtful significance. These generations are indicated by asterisks in Table III. The small numbers in the later declining generations of *H* and *Q* were unavoidable, since the fecundity of the depressed animals was very much reduced and the rate of mortality was high. Fortunately, the records of these later generations are relatively unimportant, as depression is clearly demonstrated in the preceding, larger generations. But in the test of the heritability of depression in the first *HC* and *QC* generations, the small numbers available constitute a serious complication and must be taken into account fully in the interpretation.

II. Depression: (*a*) Fecundity.

A comparison of the fecundity of the three groups of animals is presented in Fig. 4. The numbers of animals in the various generations of the *H* and *Q* series and their fecundity percentages, computed as described above, are given in Table III.

Both the *H* and *Q* groups showed some variation, but in general they had a much reduced fecundity. The egg production of the first generation of the *H* series was very low; approximately 25 per cent of the *C* average. In the next six generations there was an improvement; the fecundity of the 4th and 7th generations of *H* was even higher than that of the controls. But after that time there occurred a sharp decline, and with two exceptions, each generation had a lower productivity than the preceding one. The line died out in the 13th generation.

The *Q* series followed in general the same course as the *H* series, but with an average fecundity consistently much lower throughout the experiment. In the first generation grown in the weak culture medium, the *Q* animals produced on the average less than three per cent as many eggs as the control animals. In the next few generations they improved

and reached their maximum egg production in the 5th generation with an average fecundity of about seventy per cent of the record of the controls. From that time on there was a steady decline, each generation having a lower fecundity than the previous one. The maximum depression was reached in the 8th generation with the complete dying out of the line.

Subjecting *Proales sordida* to culture media of reduced strength for

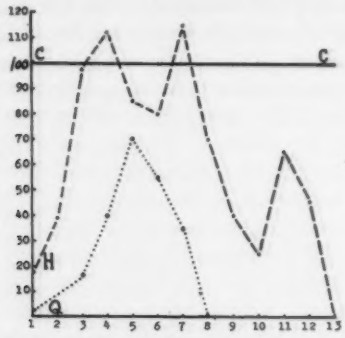

FIG. 4. Experiment I, 1928. Depression curve based on egg production. Horizontal axis represents successive generations; vertical axis the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard. In *H* the culture medium is half the strength of that in the controls; in *Q*, it is one quarter of that strength.

many successive generations is thus shown to cause a decrease in fecundity similar to that observed in other rotifers in adverse environmental conditions, and similar in many respects to the lowering of fission rate noted in poorly-fed Infusoria.

(*b*) Longevity.

There is very little in the literature on the effect of adverse conditions on longevity. Most of the environmental studies on the Rotifera present data dealing only with the effect on fecundity, considered as an index of vitality. For this reason the longevity data of these experiments are of special interest. Those for Experiment I are presented graphically in Fig. 5 in the manner already described in the consideration of fecundity. Table III gives a résumé of the records for every generation.

The effect on the length of life of the two underfed series was greater than the effect on fecundity, discussed in the last section; this is especially true of the *H* series. Neither the *Q* series nor the *H* series ever equalled in average length of life the record of the *C* group; and the records of

the three coexisting groups fall in precisely the same order, throughout their history, as that of the strengths of their culture media.

In the *H* group, the first generation lived, on the average, about sixty-five per cent as long as the contemporaneous members of the *C* group. Generations 2 to 7 showed an increase in relative length of life, rising in the 7th generation to about ninety-five per cent of the *C* record. Generation 8 showed a sharp decline which continued with two exceptions, until the 13th generation, when the line died out.

In the *Q* group the average life of the first generation was about

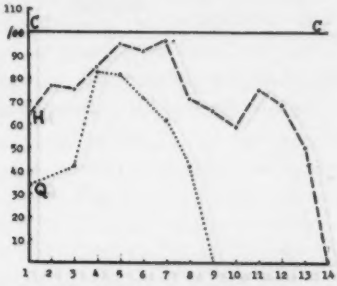

FIG. 5. Experiment I, 1928. Depression curve based on length of life. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard.

thirty-five per cent of that of the controls. The next few generations showed an improvement, and in the 4th generation the average longevity of the *Q* line practically equalled that of the 4th generation of the *H* group. From then on, however, there was a rapid decline; the line died out in the 8th generation.

These results are diverse from those obtained on the effect of alcohol by Noyes for *Proales decipiens,* and by Finesinger in most of his experiments on *Distyla inermis.* They did not find that the length of life was appreciably decreased by subjection to alcohol, even though the depressive effect on fecundity was very marked. Also, Finesinger found that in very weak doses of alcohol, both fecundity and longevity were increased. In the experiments here reported, on *Proales sordida* cultivated in inadequate media, both fecundity and longevity were strikingly decreased.

In general, then, we may say that *Proales sordida,* when grown in dilute culture media (quarter or half as strong as the optimum), and under variable temperature conditions, after an initial immediate depression, tends to adjust itself for a few generations and pursue an up-

ward course, but eventually becomes progressively depressed and dies out completely. Individuals grown in a quarter-strength solution have a lower fecundity and longevity than those grown in a half-strength medium, and in turn, individuals grown in a half-strength medium, for the most part, have a lower fecundity and longevity than those grown in a full-strength solution. In other words, the degree of depression and the speed with which it occurs are, to a large extent, dependent on the strength of the culture medium.

III. Recovery.

The question of the heritability of induced modification is of very great interest; it forms the problem of chief concern in these experiments. To test this, some of the progeny of each of the depressed groups were placed in control medium (full-strength) and their suc-

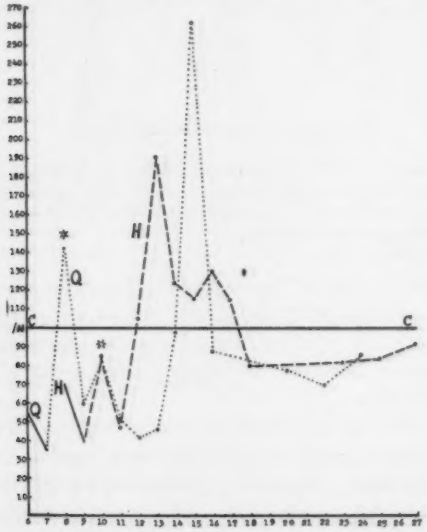

FIG. 6. Experiment I, 1928. Recovery curve based on egg production. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (C) as 100 per cent, and the records of H and Q plotted with reference to that standard. Asterisks indicate the generations in which return to normal medium occurred.

cessive generations were continuously cultivated in this medium. These "recovery groups" (HC and QC) were begun with eggs from the ninth generation of H and from the seventh generation of Q. In the curves showing the records of these groups (Figs. 6 and 7) the first genera-

tions are marked with an asterisk. All computations were made on the percentage basis described above, using the record of the controls as the standard for comparison. On the vertical scale are plotted the percentages; on the horizontal scale the number of generations. The record of the controls is indicated by the solid straight line at 100 per cent. The short solid lines preceding the dashed *HC* line and the dotted *QC* line represent the records of the generations of depressed animals (*H* and *Q*) from which the recovery groups were begun. For the detailed data on fecundity and longevity, and the numbers of animals comprising each generation, consult Table III.

(*a*) Fecundity.

At the time when the return to normal medium was made, the *H* series had an average fecundity about forty per cent of that of the control animals. As is shown in Fig. 6, the first generation in the normal medium (generation 10, marked with an asterisk) showed a considerable increase in productivity, rising to about eighty-five per cent. The next *HC* generation (generation 11) fell to almost forty-seven per cent again. The third *HC* generation (generation 12) improved markedly, with an average egg production slightly higher than that of the control series; and the next five generations, though showing a considerable fluctuation, maintained an average fecundity consistently above that of the controls. In the 9th generation in normal medium (18th generation of cultivation) the *HC* group had an average fecundity of 80 per cent and stayed at about that level for the remainder of the experiment.

The animals from the *Q* line which were placed in control medium (full-strength) also showed great variation in fecundity. Ten of the twelve *QC* generations had a fecundity lower than that of the controls. The other two (first and eighth *QC* generations) showed a fecundity much higher than that of the controls. The first *QC* generation showed an increase from 36 per cent (the record of the preceding *Q* generation, cultivated in quarter-strength culture medium) to about one hundred and forty-one per cent, exceeding the control record by a wide margin. The eighth *QC* generation (comprising only two animals) rose to 262 per cent, more than doubling the record of the controls.

For a correct evaluation of the significance of these very irregular records of the *HC* and *QC* series, the numbers of animals in the various generations (given in Table III) must be taken into account. With at most two exceptions the early generations of recovery are composed of so few individuals as to render the significance of their data doubtful. As far as they go, they indicate that recovery of egg production is immediate and complete in the first generation grown in normal culture medium.

(*b*) Longevity.

On the whole, in both test lines, variation in length of life after the return to normal culture medium was much less than variation in egg production. The data are shown in Fig. 7, plotted on the percentage basis as earlier described. The first generations in full-strength culture medium are indicated by asterisks.

The average length of life of the first generation of the *HC* series

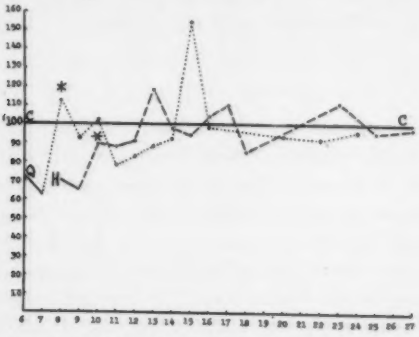

FIG. 7. Experiment I, 1928. Recovery curve based on length of life. Horizontal axis represents successive generations; vertical axis represents the percentages with the record of the controls (*C*) as 100 per cent, and the records of *H* and *Q* plotted with reference to that standard. Asterisks indicate the generations in which the return to normal medium occurred.

was considerably greater than that of the preceding *H* generation. This increase in longevity was continued for several generations, the 4th generation of recovery (13th) exceeding the record of the controls. From that point on, there was considerable variation, the average length of life of the *HC* series fluctuating around the record of the controls. Here, as in the case of fecundity, the fact that the early generations comprised only small numbers of animals makes the significance of their data doubtful.

In the *QC* series, the average length of life of the first generation exceeded the record of the controls, indicating that recovery was immediate and complete. In generations 2 and 3 (9 and 10) the longevity of the *QC* series remained very close to that of the controls; but after that, except for one generation (15th) the average was slightly lower than that for *C*. Here, also, the fact that only one (generation 10) of the first nine generations of recovery in this group was significant from a statistical point of view makes the results unreliable. However, the indication is that the decreased length of life produced by insufficient food is not inherited when the progeny are placed in an adequate environment.

Discussion

The results of these experiments show that the Rotifera do respond to an unfavorable environment as do the Infusoria; they become progressively depressed, show a gradual lowering of vitality, and eventually die out. In the adverse condition used, an inadequate culture medium, successive generations show this graded decline in both fecundity and longevity.

The results bearing on the question of the heritability of induced modifications, however, are not decisive, owing to their great irregularity. It seems probable that this irregularity was largely due to the extreme temperature variation and to the inconsiderable number of animals available in the critical generations. To control the temperature, and in the light of the experience gained to test again the heritability of the depression, a second series of experiments was carried out. An account of these follows.

Part II. Experiments of 1929

In 1929 the experimental work was conducted in a constant temperature room where fluctuations were very slight (20° to 22° C.). Variations from day to day were usually less than half a degree, and omitting the last week of the experiment when considerable fluctuations occurred, the total variation for the summer was less than two degrees.

Preliminary tests were made to find out whether cultivation under uniform temperature necessitated any change in experimental procedure. It was found that under constant temperature conditions rotifers reared in the culture medium used for the controls in the former experiments (15 flakes of oatmeal to 100 cc. spring water) had a very low fecundity and longevity and appeared to be starved. Consequently, solutions of 16, 20, 24 and 32 flakes of oatmeal to 100 cc. of spring water were tested. The 32 flake fluid was found to be the most satisfactory.

In Experiment II, therefore, the following culture media were used:

Control Series	*C*, full-strength	fluid,	32	flakes	oatmeal	to	100	cc.	spring	water
Test "	*H*, half-strength	"	16	"	"	"	"	"	"	"
" "	*Q*, quarter-strength	"	8	"	"	"	"	"	"	"

In general these media were prepared as earlier described under "Methods," except for the fact that the culture fluid was corked at the end of three hours and placed in the constant temperature room, instead of being corked at the end of twelve hours and subjected to variable temperature. It will be observed that in Experiment II the half-strength and quarter-strength culture media were twice as strong as the corresponding media of Experiment I, and that the fluid for the control series was more than twice as strong as the full-strength medium formerly

used. In all probability, the fact that the culture fluid was exposed to the air for such a short time (three hours) explains the necessity for increasing the number of flakes of oatmeal used.

General Description

This experiment was begun August 11, 1929, with a single series of control animals which were cultivated on slides for three generations before the test groups were started. These test groups, again called *H* and *Q*, were begun with some of the eggs laid by the third generation of this control series. In the 4th generation, therefore, there were three series of animals: the controls (*C*) cultivated in thirty-two flake fluid; the *H* series cultivated in sixteen-flake fluid; and the *Q* series cultivated in eight-flake fluid.

In general, Experiment II was conducted in the same way as Experiment I, dealing again with the effects on fecundity and longevity produced by continuous cultivation for many generations in weak culture media. When the animals had shown a marked decrease in productivity and length of life, the heritability of this environmental modification was tested by placing progeny from both test groups in the control medium of thirty-two flakes, and cultivating them in this full-strength medium for several successive generations.

Analysis of the Data

Since the corresponding generations of all three groups of animals occurred at the same time (except during the last part of the experiment, when the control line was sufficiently in advance of the other two groups to produce one more generation), and since the temperature conditions were satisfactorily controlled, there was no reason for comparing the groups of animals on any basis except that of corresponding generations. The results of the experiment are shown on this basis in Figs. 8 to 11. In these curves the horizontal axis represents the number of generations, using 1, 2, 3 for the preliminary series, then re-numbering 1, 2, 3, etc., beginning with the first generation to comprise three groups of animals. The vertical axis represents either the mean number of eggs or the mean length of life. The data for these curves will be found in Table IV.

I. Depression.

In this experiment, conducted under constant temperature conditions, and with stronger media than those used in Experiment I, the test lines, *H* and *Q*, did not die out with the passage of generations. When the

TABLE IV

Experiment II, 1929—Showing for the successive generations (Gen.) of each of the five series of the second experiment (Control, *H*, *Q*, *HC*, *QC*) the numbers of eggs isolated (No.), the numbers of these that did not hatch (N.H.), and the numbers that hatched (H.); and for the latter the mean numbers of eggs laid and the mean numbers of days lived. Data presented graphically in Figs. 8, 9, 10, 11.

Gen.	No.	N.H.	H.	Mean Eggs	Mean Days
Control Series (full-strength medium)					
Preliminary					
1	24	0	24	20.75	10.00
2	21	0	21	16.95	8.66
3	39	1	38	17.28	8.89
Principal					
1	48	1	47	13.02	7.67
2	52	0	52	12.34	8.17
3	41	3	38	14.00	9.13
4	40	1	39	15.38	9.01
5	40	0	40	13.87	8.17
6	54	3	51	14.31	7.93
7	30	3	27	10.48	7.62
8	34	0	34	9.23	7.26
9	30	2	28	9.53	6.94
10	35	4	31	10.19	6.30
11	29	0	29	8.13	5.79
12	28	0	28	6.46	5.32
13	30	7	23	6.26	6.19
14	39	12	27	9.03	7.35
15	32	10	22	8.59	9.00
***H* Series (half-strength medium)**					
1	50	1	49	9.97	7.34
2	52	1	51	8.25	7.58
3	43	2	41	5.63	6.76
4	39	0	39	6.74	7.55
5	40	4	36	6.97	7.09
6	43	2	41	6.04	6.71
7	51	5	46	5.21	6.71
8	42	4	38	5.63	6.82
9	41	1	40	6.22	6.13
10	39	3	36	4.36	5.97
11	42	3	39	3.82	5.33
12	42	9	33	3.54	6.12
13	36	6	30	5.53	6.71
14	27	4	23	8.52	8.50
***HC* Series (full-strength medium)**					
1(13)	38	4	34	12.50	8.67
2(14)	29	1	28	8.17	8.26

TABLE IV—*Continued*

Gen.	No.	N.H.	H.	Mean Eggs	Mean Days
		Q Series (quarter-strength medium)			
1	41	0	41	5.19	5.74
2	38	2	36	2.75	5.13
3	43	7	36	1.33	3.68
4	31	0	31	4.29	6.51
5	51	1	50	5.46	6.59
6	48	4	44	3.00	5.30
7	40	1	39	2.66	5.66
8	40	4	36	1.83	5.00
9	42	3	39	1.64	4.24
10	62	11	51	1.39	4.00
11	63	9	54	2.33	4.93
12	54	5	49	3.51	5.72
13	28	0	28	2.10	4.60
14	34	4	30	1.53	5.05
		QC Series (full-strength medium)			
1(9)	24	1	23	9.43	6.04
2(10)	50	9	41	8.85	6.48
3(11)	41	16	25	6.72	6.18
4(12)	39	2	37	12.91	8.29
5(13)	27	4	23	10.39	7.50
6(14)	31	4	27	6.03	7.14

experiment was concluded they had lived fourteen generations and, while showing some depression, they were reproducing well enough to make it seem probable that they would live many more generations, if not indefinitely.

(*a*) Fecundity.

Figure 8 presents graphically the data on egg production. It shows that in general the records of the three groups of animals ran parallel to each other, the half-strength (*H*) series regularly lower than the control series, and the quarter-strength (*Q*) series regularly lower than the *H* series. At first, the difference between the three groups was very great, but at the end of the experiment this difference was much less, especially between the *H* and *C* series. The control series showed considerable variation, but its average fecundity gradually decreased as the experiment progressed. The *H* line declined rapidly for the first three generations, and from then on continued to show a fairly constant though much less extensive decline until the 13th and 14th generations. In these last

two generations the *H* line showed a marked improvement, almost equalling the average of the controls. The *Q* line likewise declined rapidly when first subjected to the reduced culture medium, showing in the third generation the maximum effect on fecundity, when the average egg production fell to 1.33. The average fecundity stayed consistently low for

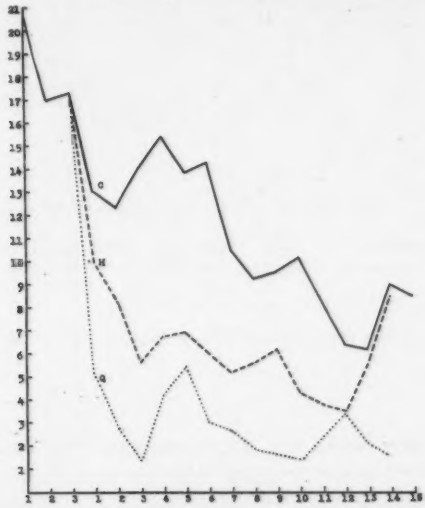

FIG. 8. Experiment II, 1929. Depression curve based on egg production. Horizontal axis represents successive generations; vertical axis, mean egg production.

the remainder of the experiment although showing considerable variation. It showed no improvement in its final generations such as the *H* series showed; the 13th generation had an average fecundity of only 2.1 eggs and the 14th generation of only 1.5 eggs. The individuals were small and thin.

Thus in this experiment, as in the former one, decreased food is associated with a decrease in fecundity, the most marked decrease occurring in the first three generations.

(*b*) Longevity.

The effects on longevity are presented in Fig. 9. The *H* series showed a moderate and gradual decline for eleven generations, but then began to improve; in the 12th, 13th, and 14th generations its average longevity exceeded that of the controls of the corresponding generations.

In the *Q* line the effect of the adverse condition was more marked,

and for the first few generations the average length of life declined rapidly, reaching its lowest point in the third generation. From that time on the *Q* line showed considerable variation but remained consistently below the other two groups except in the 12th generation, when the

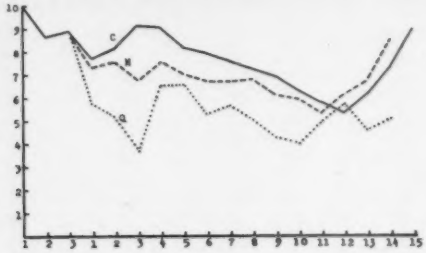

FIG. 9. Experiment II, 1929. Depression curve based on length of life. Horizontal axis represents successive generations; vertical axis, mean length of life in days.

average length of life was slightly above that of *C*, but did not equal that of *H*.

II. Recovery.

The *H* recovery series was not begun until very late in the experiment. We wished to test progeny from the most depressed generation and for that reason delayed beginning this series in anticipation of further depression in *H*, which did not occur. The *HC* series was finally started with eggs from the 12th *H* generation, a short time before the experiment terminated, and so was carried through only two generations. The *Q* recovery series was begun with eggs from the 8th generation in the quarter-strength medium. This *QC* line was continued in normal medium for six generations, when the experiment ended. The data on these *HC* and *QC* lines is presented in Figs. 10 and 11. Asterisks indicate the generations where the return to normal medium occurred.

(*a*) Fecundity.

While the *H* line in the 12th generation had an average egg production of 3.54, their progeny which were returned to the normal medium had an average of 12.5, exceeding the average of the controls by 6.24 eggs. The second (the last) *HC* generation fell to an average slightly below that of the controls.

The *Q*–*QC* series increased from an average of 1.83 eggs in the 8th generation in the quarter-strength medium to 9.43 eggs in the first generation in control medium, practically equalling the controls of that gen-

eration. They remained slightly below the controls for two generations and then surpassed them by an enormous margin in the 4th generation (twelfth under cultivation). After this they again declined to a point below the controls. The records of these two lines certainly provide

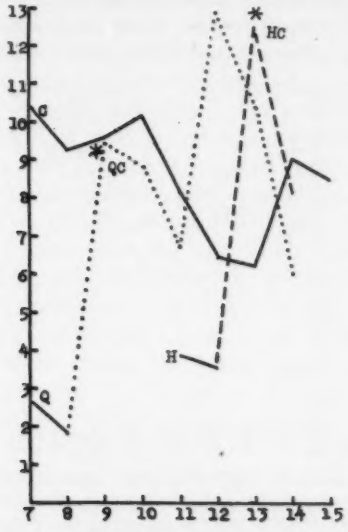

FIG. 10. Experiment II, 1929. Recovery curve based on egg production. Horizontal axis represents successive generations; vertical axis, mean egg production. Asterisks indicate generations in which return to 100 per cent medium occurred.

conclusive evidence that the effect of insufficient food on fecundity is not inherited.

(*b*) Longevity.

Figure 11 clearly shows that animals from both the test series, when placed in full-strength culture medium, recovered a normal longevity at once, their records even exceeding that of the control animals. The record of the first *HC* generation exceeded by the same amount the record of their parents (the last *H* generation) and the record of the corresponding generation of the *C* series, and fell only slightly in their second and last generation.

The longevity of the first generation of the *QC* line, grown in the full-strength medium, almost equalled the record of the controls; the four generations following exceeded the controls, and the sixth (the last) generation was only very slightly below the controls.

What has been said concerning the inheritance of decreased fecundity is likewise true for longevity. Animals whose length of life has been altered by long cultivation under adverse conditions produce progeny that show a normal longevity when grown in a favorable environment. Depression produced in this rotifer by the action of an inadequate culture medium lasts only as long as the stimulus which produces the depression is present. This result differs a little from the results of Whit-

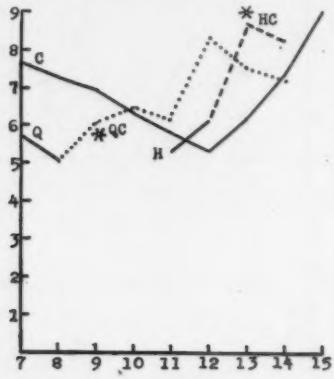

FIG. 11. Experiment II, 1929. Recovery curve based on length of life. Horizontal axis represents successive generations; vertical axis, mean length of life in days. Asterisks indicate generations in which return to 100 per cent medium occurred.

ney (1912) and Noyes (1922), who found that the depression produced by the action of alcohol persisted for two generations after removal to a favorable medium.

Mechanism of the Effect

As to the precise way in which the environment operates to produce depression, we have no evidence. It may be that in a medium deficient in food, the rotifer grows at the expense of its own productivity; that in the ordinary metabolic processes it uses up part or all of the yolk stored in the vitellarium, making the production of many eggs impossible, thus bringing about a depression in fecundity. This process may be correlated with the length of life of the organism to the extent that when all the stored yolk has been used up in metabolism, the animal has no more reserve on which to depend and consequently dies, thus bringing about a lowering in longevity. Or, on the other hand, in such a weak food medium the animal may use all the food it receives for its own body metabolism and not have enough left to produce yolk for many eggs, causing a reduction in fecundity but not in longevity, the animals

having sufficient vitality to continue living for the usual length of time if they do not use part of their energy in egg production.

It is possible that the amount of stored yolk is reduced in ill-fed animals, and that the eggs are smaller. If this were a cumulative process, the eggs of consecutive starved generations becoming progressively smaller, containing less and less yolk, the lines would eventually die out. Measurements need to be made to determine whether there is any size difference between the eggs of normal parents and the eggs of depressed parents. As far as we could determine by superficial observation, the eggs of all generations of all three groups were normal in every way.

No measurements were made on the animals in our experiments, but some of the observations on comparative size and general appearance are of interest. In the second experiment, particularly, the difference in appearance between animals belonging to different lines was very striking. The controls were large and healthy in appearance from the first; the animals of the *H* series very soon became slightly smaller than the controls, and quite transparent; the *Q* individuals were extremely small and thin. For several generations one could recognize at once to what line an animal belonged merely by its size and type. As the experiment proceeded, however, the *H* and *Q* animals improved in appearance to such an extent that in the later generations they seemed as healthy and normal as the control animals. In the last two generations some of the control animals looked bloated and heavy, and died containing dark masses of material, and often one or two or even three well-developed eggs which they had been unable to lay.

In both of the experiments, animals were observed with internal eggs which they were unable to eject; in numerous cases the egg developed within the female and hatched within her body after her death. Some of these young finally escaped from the parental corpses and lived thereafter as normal individuals, apparently not differing from animals that had hatched normally. A single instance was observed where two such young hatched out within the same female and later became liberated. This behavior was not confined to one group or one generation, but instances were observed in *C*, *H*, and *Q*, in the later generations of both experiments.

Summary and Conclusions

This paper is a study of depression and recovery in the parthenogenetic rotifer, *Proales sordida* Gosse, showing the response of the organism to various dilutions of culture medium. All the animals used were members of a single clone and hence were genetically alike. Oatmeal infusions of half and quarter-strengths, as compared with the full-strength medium used for the control animals, were employed. The in-

vestigation consisted of two series of experiments conducted separately one year apart, together with some supplementary procedures. It was found that with continuous parthenogenetic reproduction, rotifers in the reduced strengths of culture media become depressed, decline in vitality with the passage of generations, showing a much lowered fecundity and longevity. Under conditions of variable temperature this decline is cumulative and results in the ultimate death of the lines. Under constant temperature conditions the animals show a cumulative decline in the first few generations, reaching a low level of fecundity and longevity, where they remain without further depression. The degree of depression and the speed with which it occurs depends on the strength of the medium employed. This depression produced by means of an environmental agent is similar in many ways to that produced by the action of adverse conditions in various Infusoria and in other rotifers. However, in contrast to the results obtained by others in studies of the Rotifera, longevity as well as fecundity is affected by the environmental agent.

The progeny of the depressed animals recover their normal fecundity and longevity at once in the first generation placed in a favorable medium. Apparently the food deficiency does not affect the germ cells so as to produce a heritable modification.

The protozoan constitution seems to be extremely sensitive to environmental modifications and becomes changed to such an extent that the alterations are passed on to the progeny of later generations. Some of these changes are adaptive, so as to cause an increase in resistance to certain agents, while others (such as those resulting from food insufficiency) are degenerative and result ultimately in the extinction of the race. The situation in the Rotifera, as apparently in the other Metazoa, is quite different. Though in Rotifera it is possible to produce and maintain depression for a long period of time, and though the depression becomes greater in later generations, it disappears at once, or within one or two generations, upon restoration to normal conditions. The complete agreement in this respect of the results of the present study with those of Whitney, Noyes, and Finesinger on the injurious effects of alcohol goes far to establish this as a definitive conclusion for the Rotifera. Injurious conditions acting on the individuals of many successive generations and producing a progressive decline, do not alter the germinal materials carried by these individuals.

It is possible that this difference between the Rotifera and the Protozoa may be related to the differences in the details of their reproductive processes. In the Protozoa the body divides into two equal parts; each part becomes a new individual. In *Proales,* as in most Metazoa, a smaller piece separates from a larger. The larger piece is called the

parent; the smaller the egg. This smaller piece goes through a twenty-four-hour-long process of development, including numerous cell divisions, into a new *Proales* individual. It does not seem obvious *a priori* that this difference in detail must result in diverse genetic behavior. These experiments on the heritability of an environmental effect indicate that they do.

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THE RÔLE OF THE BASAL PLATE OF THE TAIL IN REGENERATION IN THE TAIL-FINS OF FISHES (*FUNDULUS* AND *CARASSIUS*)

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Experiments on the tails of fishes from a standpoint of morphogenesis have been performed by Morgan (1900, 1902 and 1906), and by the writer (1929). A review of the literature may be found in Nabrit (1929).

The writer suggested in the previous paper, after comparing the findings of Harrison (1918) and Detwiler (1918) in *Amblystoma* with the results of his experiments in fishes, that a possible similarity existed between the production of limbs in *Amblystoma* and tail-fins in fishes. Each one seems to be an independently differentiating mesenchymal system.

It was further concluded that the rate of regeneration from cut surfaces in the tail-fins of fishes is regulated by the cross-sectional area of the fin rays exposed.

Broussonet (1786), Morgan (1906), and Morrill (1906) agreed that ray stumps must be left for regeneration to take place. Morrill suggested that regeneration does not take place when the remaining stumps are too small. This point was left open for further investigation by the writer and is considered in this paper.[1]

The experiments were carried out during the summer of 1929 at the Marine Biological Laboratory at Woods Hole, Massachusetts, and during the winter of 1929–1930 at Morehouse College. At Woods Hole, *Fundulus heteroclitus* was used and at Morehouse College, the goldfish, *Carassius auratus*. In both cases, adult animals were used.

Rays were carefully picked from the tails by fine forceps. The tail and the rays were examined under a binocular microscope to make sure that no parts of the rays were left embedded in the tails.

After regeneration was complete, examinations were made upon the living and then upon the fixed tails.

In three weeks after removing the fin rays, undifferentiated mesen-

[1] I wish to express my sincere thanks to Professor J. Walter Wilson of Brown University for his very helpful suggestions and criticisms in this investigation.

chyme had filled in the gap made in the tails. In from four to six weeks, rays appeared in the regenerated mesenchyme (*a*, Figs. 1 and 2). These rays begin at the basal plate and differentiate distally. They segment and may branch as any other rays in the tail, though some rays do not branch (*b*, *c*, Figs. 1 and 2). If they branch at all it is usually dur-

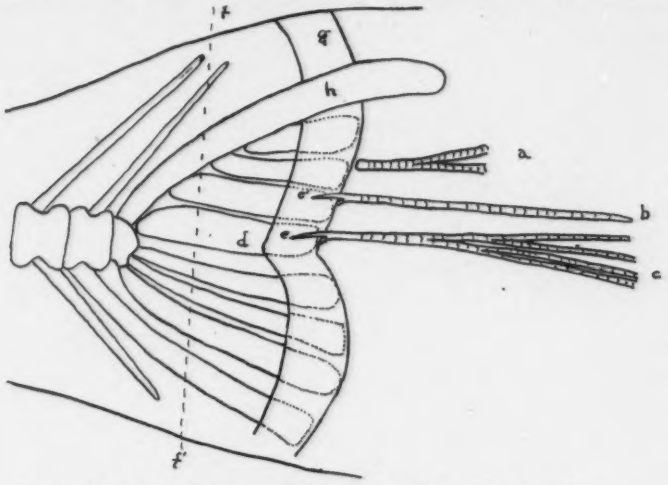

FIG. 1. *Carassius.* Diagrammatic sketch of the base of the tail.
a. A newly regenerated ray (six weeks).
b. A ray six months after it started regeneration. It did not branch.
c. A regenerated ray after six months.
d. Basal plate.
e and *e'*. Proximal end of fin ray.
ff'. Region of a cut anterior to proximal end of fin rays.
g. Articulating region of basal plate.

ing the first two months. The anterior knobs or proximal ends of the rays are completed in from five to six months (*e*, *e'*, Figs. 1 and 2). The proximal ends are produced on both sides of the basal plate.

In the embryonic development of the tail in *Fundulus*, the rays streak out from a common mesenchymal plate. They begin to appear between the ninth and thirteenth days. From two to four appear at a time, dorsally and ventrally placed in respect to the previously formed rays.

When a cross-cut is made in the tail anterior to the proximal knobs of the fin rays (*ff'*, Figs. 1 and 2), regeneration does not readily take place. This led to the conclusion that the ray stumps must be left in the tail for regeneration of the tail to occur.

When rays are picked out of the tail, they readily regenerate; the

rate, however, is slower than it would be if ray stumps were present. It would appear, therefore, that no parts of the rays are necessary for regeneration.

New rays do not begin to appear at their original most proximal point, but at the distal end of the basal plate. Physically this is necessary unless two separate anlage are developed for each ray, as each ray has a component on each side of the basal plate.

When a cross-cut is made anterior to the proximal knobs of the fin rays, the cut also includes the anterior articulating portion of the basal

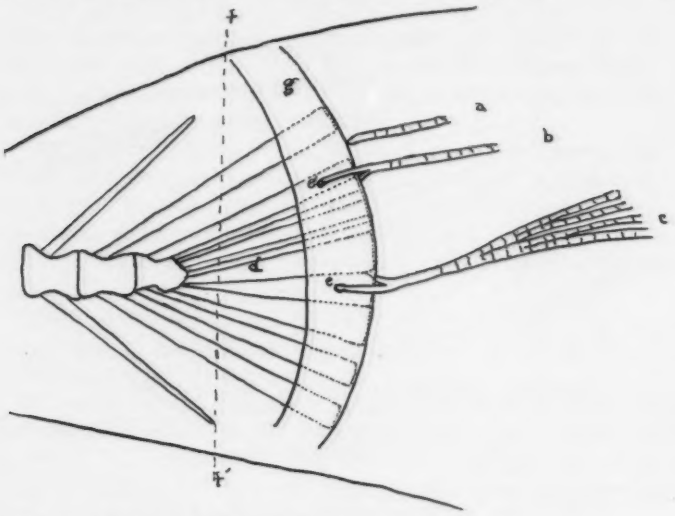

FIG. 2. *Fundulus.* As above.
a. New ray, three and one-half weeks.
b. A ray, six and one-half weeks.
c. A normal ray.
d. Basal plate.
e and *e'*. Proximal end of fin ray.
ff'. Region of a cut anterior to proximal end of fin rays.
g. Articulating region of the base of tail.

plate. In such a case the delay in regeneration would be so great that the animal would be cast aside, even if, as is usually the case, the flesh did not slough off and lead to death.

A cut ray will regenerate those parts distal to the level of the cut. An anterior knob regenerates the entire ray. Axial heteromorphosis on the knob has not occurred. But when an anterior knob is severed or removed from the distal stump, axial heteromorphosis occurs in the dis-

tal portion. When a ray is picked out, on the other hand, the new distal portion appears at the end of the articulating portion of the basal plate. Now instead of getting axial heteromorphosis with this distal portion, there is added a normal anterior knob. The visible difference in these two cases is that this new ray is in connection with the basal plate. It thus seems evident that the basal plate gives rise to the parts distal to it, or induces their development. This suggestion gains further support from the fact that the articulating part of the basal plate is shaped like the tail in *Carassius* and in *Fundulus,* bilobated in the former and rounded in the latter, and may still retain the original formative influences which cause the differentiation of the fin rays and hence the size and form of the tail.

It seems that a further similarity is demonstrated between the morphogenesis of tail-fins of fishes and the *Amblystoma* limbs. The articulating plate, like the girdle, may give rise to parts distal to it. In regeneration as in embryonic development, the basal plate gives rise to or induces the development of rays. The rays, like the limbs in *Amblystoma,* and the basal plate, like the girdle, belong to a self-differentiating mesenchymal system.

Summary

1. By picking out the fin rays instead of cutting anterior to them, it is shown that ray stumps are not necessary for regeneration in the tail-fins of fishes.

2. When the stumps are removed it seems that the new rays appear under the influence of the articulating portion of the basal plate of the tail.

3. From the embryological development and mode of regeneration a similarity is demonstrated between morphogenesis of limbs of *Amblystoma* and the tails of fishes, each being a self-differentiating mesenchymal system.

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RELATIVE RESISTANCE OF FOURTEEN SPECIES OF PROTOZOA TO THE ACTION OF *CROTALUS ATROX* AND *COBRA* VENOMS

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Essex and Markowitz (1930) have offered data which lend support to the conception that *Crotalus* venom is a non-specific protoplasmic poison that affects protoplasm wherever it comes in contact with it. This is in contrast with the view that snake venom is a complex of various constituents, each specific for certain kinds of cells. In a recent publication (1930) I have presented data, on eleven species of Protozoa, which indicate a wide difference in the degree of susceptibility to the effect of *Crotalus atrox* venom. Some species were not affected by the concentration of the venom used (0.00025 gram of dry venom per cc. of medium), while some were killed quickly. No attempt was made, however, to determine whether the races of Protozoa found to be resistant would be affected by higher concentrations of the venom. In the light of the conclusions of Essex and Markowitz concerning the general nature of the poisonous effects of *Crotalus* venom it has seemed advisable to continue the investigation of the effect of venoms on Protozoa to determine if any species is wholly resistant to their destructive action. The investigation is continued also as a phase of a more extended study, which is in progress, of the resistance which races of Protozoa acquire to venoms.

For each of the fourteen species of Protozoa used in this investigation a determination has been made of the least concentration of *Crotalus atrox* and of *Cobra* venom required to kill the animals within one hour. This, for each species, is called the minimal lethal concentration. The method used in determining this minimal lethal concentration is the same as that described in a previous publication (1930). The minimal lethal concentration of each venom for each of the species of Protozoa is shown in the accompanying table.

An inspection of the data shown in the table reveals the fact that the venoms at some concentration have a lethal effect on each of the species of Protozoa used. It is evident, however, that the Protozoa

Minimum Lethal Concentration of Crotalus atrox and Cobra Venoms for Fourteen Species of Protozoa

Species of Protozoa	Minimal Lethal Concentration	
	Cobra Venom, gm. per cc.	Crotalus atrox Venom, gm. per cc.
Amœba dubia	.000008	.001500
Blepharisma undulans	.000015	.000100
Chilomonas paramecium	.000010	.000300
Coleps hirtus	.000020	.000900
Colpidium campylum	.000006	.003000
Euglena gracilis	.000040	.000200
Euplotes patella	.000010	.000150
Oxytrichia fallax	.000040	.000150
Paramecium multinucleatum	.000010	.000050
Prorodon teres	.000025	.000300
Stentor polymorphus	.000200	.000100
Trachelius ovum	.000060	.000900
Urocentrum turbo	.000004	.000100
Vorticella sp.	.000100	.002000

exhibit a great variation in respect to the degree of the resistance of each species to the venoms. All species are more resistant to *Crotalus atrox* venom than to *Cobra* venom. Some species (*Urocentrum, Paramecium, Euplotes, Blepharisma*) are, in relation to other species, highly susceptible to both venoms. One species (*Vorticella*) has relatively great resistance to both. Some species (*Colpidium, Amœba, Chilomonas, Coleps* and *Prorodon*) are highly resistant to *Crotalus* venom but quite susceptible to the effects of *Cobra* venom. On the other hand, one species (*Stentor*) is, in relation to the other species, highly resistant to *Cobra* venom but susceptible to the action of *Crotalus* venom. The same is true to some extent with *Trachelius*.

Repeated titrations made in connection with previous studies have shown that the minimal lethal concentration for *Paramecium multinucleatum* of *Cobra* venom and of *Crotalus atrox* venom is respectively 0.0000016 gram and 0.00002 gram per cc., while in this study it is, respectively, 0.00001 gram and 0.00005 gram per cc. This indicates that the stock solution of *Cobra* venom used in this investigation was approximately one-sixth normal strength, while that for *Crotalus atrox* venom was two-fifths of its normal strength. Repeated tests were made with *Paramecia* to show that the stock solutions of the venoms used throughout this study did not deteriorate in strength during the time. The stock solution in each case consisted of 0.05 gram of dry venom dissolved in 4.5 cc. of distilled water to which 0.5 cc. of glycerine was added. The stock solutions were rendered neutral by the addition of Na_2HPO_4.

It may be concluded that, so far as this group of Protozoa is concerned, the poisonous effect of *Crotalus atrox* venom and of *Cobra* venom on Protozoa is general. There exists among the species, however, much variation in the resistance to the action of the two venoms, some species having relatively high resistance to the one and low to the other, while, in other species, the reverse relationship exists.

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A DETERMINATION OF THE TENSION AT THE SURFACE OF EGGS OF THE ANNELID, *CHÆTOPTERUS*

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By means of the microscope-centrifuge, recently described by Harvey and Loomis (1930), it is possible to observe, and to photograph, using highest dry objectives, living cells while subjected to centrifugal forces thousands of times gravity.[1] The cells are mounted on a special slide fixed over the objective, which rotates on the centrifuge at a distance of 11 cm. from the axis of rotation. By total reflecting prisms the image is brought to the axis of the centrifuge and then vertically upward where it is observed with a stationary ocular. The light, a condenser discharge in mercury vapor, is arranged for stroboscopic illumination, flashing on with each revolution of the centrifuge. The image is perfectly clear and steady at 4000 R.P.M.

Chætopterus eggs, as is well known from the work of F. R. Lillie (1909), pull apart into fragments under the influence of high centrifugal forces. The centrifuge-microscope allows us to observe clearly the series of changes in this process and to make exact measurements from which it is possible to calculate the tension at the surface of the egg. This process is illustrated for unfertilized *Chætopterus* eggs by the photographs of Fig. 1.

The first three rows show ten stages in the formation of a fragment or spherule containing oil, taken through the microscope-centrifuge while revolving about 4000 R.P.M., 5 seconds' exposure. It will be noted that No. 5 is a double exposure (by mistake) and shows the exact change in position which takes place in 1 minute. At one stage the protoplasm is drawn out into long filaments with the oil spherule at one end. In the pulling off of this oil spherule the appearance is that of a drop of molten glass slowly falling from a heated rod. When the oil finally breaks away, it moves slowly (as long as it can be observed in

[1] The microscope-centrifuge was devised in collaboration with Mr. Alfred L. Loomis and constructed in his private laboratory at Tuxedo Park, New York. The author expresses his deep appreciation to Mr. Loomis for the hospitality of the laboratory. It is contemplated that the instrument will be placed on the market by the Bausch and Lomb Company. For description see Harvey and Loomis in *Science*, 1930, **72**: 42.

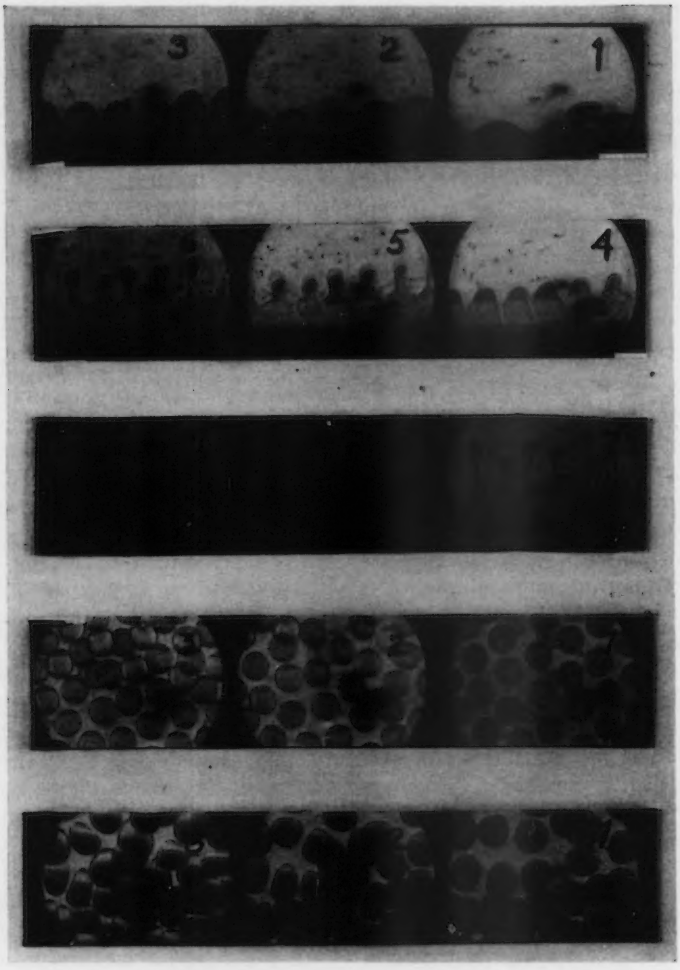

FIG. 1. Photographs of unfertilized eggs taken with a Leica camera through the microscope-centrifuge (radius 11 cm.), showing successive changes (examine from right to left) in stratification of granules and in shape of eggs. Magnification, 87 diameters. The direction of the centrifugal force is downward.

First three rows, *Chætopterus* exposed for 5 seconds at 35, 55, 95, 165, 205 and 265 (double exposure), 345, 375, 450, and 480 seconds after starting centrifuge. Speed of the microscope-centrifuge was 55 R.P.S. in the first row and 66 R.P.S. in the second and third rows. At the highest speed about 330 flashes of light occurred during the exposure.

Fourth row, *Cumingia* exposed 10 seconds at 35, 95 and 155 seconds after starting centrifuge. Speed 38 to 55 R.P.S. The eggs are separated by jelly.

Fifth row, *Arbacia* exposed 10 seconds at 45, 150, and 285 seconds after starting centrifuge. Speed 35 R.P.S. The eggs are partially separated by jelly.

the field of the microscope) to the surface of the sea water. Sometimes a clear fragment containing no yolk or oil globules will separate. These clear fragments are denser than sea water and sink; in fact, a clear fragment, one half containing oil globules, is heavier than sea water.

The pulling away of an egg spherule containing oil occupies about seven minutes at 1916 times gravity, and it will be noted that when an oil spherule breaks away the stalk does not round up immediately but remains for some time and only slowly rounds off. The stalk does not behave as if it were elastic like rubber, which would immediately recover when stretched, nor is it stiff, for one can observe these stalks waving back and forth in currents in the sea water during centrifuging. They appear like a very slow flowing plastic material such as tar.

The stalk is part of the original surface of the egg. Are we to regard that as having purely a surface tension at its boundary or a membrane of some sort with an elastic tension and a definite breaking strength, or should we think of the breaking strength of the stalk per cross sectional area? Perhaps the magnitude of this tension will allow us to decide. If we determine the buoyant force of the oil tending to draw apart the egg into fragments, we can equate this to a tension of the egg considered as acting only around the surface (surface tension or elastic tension of a membrane) or as acting over the whole cross sectional area of the stalk (internal friction of a plastic fluid), or a combination of the two.

Fortunately, it is easy to gain a rough idea of the buoyant force, which tends to pull apart the egg, by the following considerations. A maximum value for this force will be obtained if we consider oil rising through egg fluid rather than egg spherule rising through sea water. Let us assume the density of the oil is 0.915, the same as that of hen's egg yolk oil. By measuring the diameter of the oil spherule ($34\,\mu$) pulled away from the egg, we find that the volume of the oil is 75 per cent[2] of 2.05×10^{-8} cc. or 1.54×10^{-8} cc., and its mass is 1.41×10^{-8} grams. The density of the medium can be taken as 1.044, the average of Heilbrunn's (1926) values for *Arbacia* and *Cumingia* eggs, and slightly greater than sea water (1.0238) at Woods Hole at 20° C. Therefore, the buoyant force of the oil pulling inward toward the axis of the centrifuge will be $(1.044 - 0.915)/0.915 \times 1.41 \times 10^{-8} = 0.2 \times 10^{-8}$ grams $\times$ 980 $\times$ centrifugal force $(1916 \times g) = 3.75 \times 10^{-3}$ dynes. Since the yolk rests against the bottom of the slide, this will be the only force tending to pull the egg apart, and it will be counter-

[2] Since the oil granules are spheres and spheres packed in a volume occupy only 75 per cent of that volume.

acted by the surface tension or elastic tension (T) of the egg, acting around the circumference (πD) of the stalk of diameter (D) connecting oil spherule and egg at the moment the stalk breaks.[3] Hence $\pi DT = 3.75 \times 10^{-3}$ dynes.

The diameter of the stalk is found to be 9 μ by measurement. Solving, $T = 1.32$ dynes per cm. This is a maximum value, as it is likely the oil might have been pulled away with somewhat lower centrifugal forces, although it takes 7 minutes to pull apart at $1916 \times$ g. One dyne per cm. cannot be very far from the truth, perhaps 25 per cent error at most. If we are to regard this as a surface tension, it is very low, but might be compared to that of a water—isobutyl alcohol interface whose T is 1.76 dynes per cm. at 20° C. On the other hand, the value seems entirely too low for an elastic membrane tension.

If we regard the stalk of *Chætopterus* eggs as made up of homogeneous material, its breaking strength, S, would be $(\pi/4)D^2S = 3.75 \times 10^{-3}$ dynes or $S = 5900$ dynes per cm.^{2}, also an extremely small value. The breaking strength of rubber is about 100 million dynes per cm.^{2}

The long cylindrical stalk of centrifuged *Chætopterus* eggs would be a very unstable figure according to classical surface tension interpretation. Plateau showed that a cylinder breaks into drops when its length equals its circumference. This is a geometrical relation, independent of the value of the surface tension, provided the fluids have a low viscosity. In the derivation of surface tension formulas by the vibrating drop and vibrating jet methods the viscosity of the fluid is neglected for simplification. With very high viscosities the attainment of equilibrium conditions might take so long a time as to make thin cylinders stable figures for all practical purposes. With plastic fluids surface tension might be too small to overcome the internal friction.

The value of 1.3 dynes per cm. does not allow us to decide definitely whether the boundary of the egg exerts a surface tension or an elastic tension, but it does show that there can be no very firm "pellicle" around these eggs. The observed behavior of the stalk makes it quite certain that we are dealing with a very plastic material. If the tension were greater than fifty dynes per cm., a maximum value for liquid-water interfaces, we might say quite definitely that it was elastic membrane and not surface tension. Such may be the case in eggs like *Arbacia* and *Cumingia* which are not torn apart by much higher centrifugal forces, and show only a slight tendency to lengthen, as illustrated in rows 4 and 5 of Fig. 1.

[3] I am deeply indebted to Dr. Charles Zahn of the Physics Department, Princeton University, for suggestions regarding the interpretation of some of these surface tension phenomena.

SUMMARY

A method is described, using the microscope-centrifuge, for calculating the tension at the surface of *Chætopterus* eggs, which gives an approximate *maximum* value of 1.3 dynes per cm.

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THE EFFECT OF CERTAIN NARCOTICS (URETHANES) ON PERMEABILITY OF LIVING CELLS TO WATER

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The experiments on narcotics reported in this paper were carried out with the purpose of gaining further information on the factors which regulate or influence permeability of the living cell to water. Former studies on sea urchin eggs have shown that cell permeability to water is not constant, but varies with a number of factors, an important one being the chemical composition of the medium (McCutcheon and Lucké, 1928). Thus permeability is regulated, at least in part, by the sign and the number of charges on the ions of the medium, in the sense that anions increase and cations decrease permeability to water; the effects are the greater the higher the valence of the ions (Lucké and McCutcheon, 1929). In the presence of cations of two or more valences permeability is of very low magnitude. For example, in hypotonic sea water cells, such as unfertilized eggs of the sea urchin, have a numerical value of permeability of approximately 0.05 at 15° C., *i.e.*, 0.05 cubic micron of water pass through each square micron of cell surface per minute, under the driving force of one atmosphere of osmotic pressure.[1] This surprisingly low degree of permeability is presumably due to the high concentration of calcium and magnesium in sea water, for the same low value of permeability is obtained when cells are placed in a non-electrolyte medium, of like osmotic pressure, containing as little as 0.0001 molar $CaCl_2$ or $MgCl_2$. But all attempts to further decrease permeability have so far been unsuccessful. However, several writers have reported that narcotics decrease permeability to water (Winterstein, 1916; Lillie, 1918; and Anselmino, 1928). The question therefore arose whether narcotics in the presence of sea water, or narcotics in any solution containing cations of two or more valences, would lower permeability beyond the value obtained in sea water alone.

METHOD

A satisfactory method of studying quantitatively the effect of various factors on permeability to water is to place a suitable cell in a

[1] By permeability to water is understood the quantity of water passing through unit area of cell surface in unit time under unit pressure.

hypotonic medium, thus causing water to enter the cell under the driving force of osmotic pressure. The spherical unfertilized egg of the sea urchin, *Arbacia punctulata*, is an excellent natural osmometer (McCutcheon and Lucké, 1926; McCutcheon, Lucké and Hartline, 1931 *a* and *b*). When placed in a hypotonic solution it swells relatively slowly, thus permitting accurate measurement of its diameter, from which volume and surface area can be calculated; it has a high degree of semipermeability, retains its spherical shape during change of volume, and allows ready determination of injury which may have taken place during the experiment.

The narcotics selected for this study are certain urethanes and carbamates.[2] These compounds have the advantage of being non-injurious over a considerable range of concentration; they are non-volatile, penetrate almost instantaneously, and their narcotic effect is easily demonstrable with the material used.[3]

In the first series of experiments the narcotics were dissolved in ordinary (100 per cent) sea water. Unfertilized eggs of *Arbacia* were exposed to these solutions for from 5 to 10 minutes. The cells were then transferred to hypotonic sea water (usually 40 per cent sea water) with the narcotic to be tested in the same concentration as in the isotonic solution. The course of inflow of water at constant temperature ($15 \pm 0.5°$ C.) was then observed by measuring the diameter of three cells at minute intervals with a filar micrometer for six successive minutes.[4] The mean volume of the cells was plotted against time and a smooth curve drawn through the points. The rate of passage of water is given by the rate of change of volume, dV/dt, and is obtained from the slope of the curve at a given time, t. The numerical value of permeability to water is calculated from the equation:

$$\text{Permeability} = \frac{dV}{dt} / S(P - P_{ex}),$$

where S is the surface area of the cell and $(P - P_{ex})$ the difference in pressure between the interior of the cell at time t and the medium.[5]

[2] For the sake of brevity the compounds will be referred to in this paper as urethanes. The propyl urethanes were not commercially obtainable, and were kindly supplied by Dr. Ralph Major, then of Princeton University.

[3] The narcotic effect of these compounds will be dealt with by Dr. E. B. Harvey in a forthcoming paper.

[4] The technic of measuring volume changes in *Arbacia* eggs by means of a filar micrometer eye-piece has been previously described (McCutcheon and Lucké, 1926).

[5] For details of calculation see McCutcheon and Lucké, 1928, 1929, and Lucké, Hartline and McCutcheon, 1931*b*.

Effect of Urethanes When Dissolved in Sea Water

Before determining the effect of these narcotics on permeability a preliminary question needed to be answered: Do these compounds affect the volume of cells in equilibrium with either an isotonic or hypotonic medium? If they should not enter practically instantaneously the volume of the cells would decrease when transferred to isotonic sea water containing a considerable concentration of the narcotic (*e.g.*, 0.2 m. ethyl urethane). On the other hand, if these compounds do enter rapidly, but cause severe injury or death, the volume of the cell in equilibrium with a hypotonic solution might be either too small (from escape of dissolved substances) (Lucké and McCutcheon, 1930) or too large (from splitting of substances in the interior of the cell) (Lucké and McCutcheon, 1926). Now, in order to use the simple equation for permeability given above it is necessary that the equilibrium volume of the cells in the solution to be tested should be the same as the volume at equilibrium of control (unnarcotized) cells in both isotonic and hypotonic concentrations of their natural medium, sea water. This question was answered as follows:

Different lots of cells from the same animal were placed in sea water in which had been dissolved a narcotic in the concentrations shown in Table I. After 5 to 10 minutes' exposure a number of cells were meas-

Table I

Effect of Urethanes on Cell Volume at Equilibrium

The concentrations shown are the highest used in the experiments. V_o is the mean volume of 25 cells measured after from 5 to 10 minutes' exposure to the narcotic solution in isotonic (100 per cent) sea water, V_e is the mean volume of 25 cells in equilibrium with a given hypotonic solution in which were dissolved the different urethanes (measured 4 hours after transfer). It is seen that the volume of cells in the narcotic solutions corresponds closely with the volume of control cells in the same concentration of sea water. The figures must be multiplied by 100 to obtain volumes in cubic micra.

Solution	V_o	V_e
First sea water control	1908	3255
Second sea water control	1882	3230
Ethyl urethane 0.2 m	1933	3182
n-propyl urethane 0.1 m	1920	3230
i-propyl urethane 0.1 m	1876	3182
n-butyl carbamate 0.05 m	1884	3275
Phenyl urethane 0.002 m	1924	3273

ured and then transferred to a hypotonic solution containing the same concentration of the urethanes. The volume at equilibrium was

determined after 4 hours' exposure at 22° C. Table I shows the result of a representative experiment. It is seen that there is no change in volume of the narcotized cells in isotonic sea water, and the final equilibrium attained in hypotonic solutions corresponds to that of the control (unnarcotized) cells.[6] Other experiments gave similar results.

It was, therefore, possible to study the effect of these narcotics on cellular permeability to water by the method outlined above. In Table II are given three experiments, representative of a larger number. The table shows that none of the urethanes over a considerable range of definitely narcotic concentration caused significant change in permeability from the controls, excepting that increase in permeability occurred when the cells became injured during the experiment.[7]

These and similar experiments lead to the conclusion that urethanes in narcotic concentration when dissolved in sea water do not decrease permeability of the living cell to water.

Effect of Urethanes When Dissolved in a Non-Electrolyte Medium

The result obtained with narcotics when dissolved in sea water, *i.e.*, failure to decrease cell permeability to water, may possibly be explained on the grounds that at a given temperature permeability can be reduced only to a certain value, and that this value is the one normally obtained in sea water. Since, then, the presence of the bivalent cations of sea water might, perhaps, mask the action of narcotics, the experiments were repeated in hypotonic dextrose solution.

The experiments were carried out as follows: Unfertilized eggs of *Arbacia* were washed in 0.95 molal solution of dextrose, isotonic with sea water, to eliminate electrolytes from the medium. The cells were then caused to swell in 0.38 molal dextrose solution, isotonic with 40 per cent sea water. Other eggs from the same animal were washed in isotonic dextrose solutions containing in the one case urethane, in the other calcium chloride; they were then measured during the course of swelling in hypotonic dextrose solution to which had been added ure-

[6] It should be pointed out that *prolonged* exposure to urethane solutions may change the shape of cells. This is especially the case in the more concentrated solutions of ethyl and propyl urethane, in which the normally spherical cells may become transformed to bizarre amoeboid forms. No such changes occurred when cells were exposed to less concentrated solutions, for a shorter length of time and at a lower temperature. In the experiments here reported, cells retained their normal shape.

[7] The narcotics, in the experiments here reported, were used in three different concentrations, of which the lowest was still definitely narcotizing and the highest not toxic under the conditions of the experiment. Still higher concentrations proved injurious and increased permeability.

The narcotizing effect was kindly determined by Dr. E. B. Harvey on the basis of cleavage experiments.

TABLE II

Urethanes in Sea Water

In these three experiments the narcotics in the molar concentrations shown are dissolved in 40 per cent sea water (40 parts of sea water and 60 parts of distilled water). In the last column of the table is given the value of permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was 15 ± 0.5° C.

It is seen that the various urethanes do not decrease permeability beyond the value obtained in sea water alone. The increased values of permeability (indicated by an asterisk) were obtained in injured cells, *i.e.*, in cells which failed to cleave when returned to ordinary sea water and inseminated at the conclusion of the experiment.[8]

Compound	Concentration	Permeability
Ethyl urethane	0.2	0.055
" "	0.1	0.057
" "	0.05	0.056
First sea water control		0.054
Second sea water control		0.054
n-propyl urethane	0.1	0.096*
n-propyl urethane	0.05	0.052
n-propyl urethane	0.025	0.056
i-propyl urethane	0.1	0.057
i-propyl urethane	0.05	0.051
i-propyl urethane	0.025	0.055
First sea water control		0.058
Second sea water control		0.053
n-butyl carbamate	0.05	0.089*
n-butyl carbamate	0.025	0.061
n-butyl carbamate	0.0125	0.063
i-amyl carbamate	0.01	0.059
i-amyl carbamate	0.005	0.061
phenyl urethane	0.005	0.060
phenyl urethane	0.0025	0.057
phenyl urethane	0.00125	0.056
First sea water control		0.059
Second sea water control		0.069

[8] It has previously been shown (Lucké and McCutcheon, 1930) that injury induced by high temperature and by anisotonic solution causes an increase in cellular permeability to water. From the experiments given in the table as well as from similar experiments, it is evident that injury induced by toxic concentrations of narcotics also increases permeability to water. Higher concentration of narcotics than those employed produced injury, and hence increased permeability.

thane or calcium, in the same concentration as was present in the isotonic solution of dextrose. Permeability was therefore determined, in each experiment, in pure dextrose solution, in dextrose solution containing a narcotic, and in dextrose containing calcium.

The results of three such experiments are shown in Table III. It is seen that the values of permeability in pure dextrose solution are about twice as great as in calcium-dextrose solution, while permeability values

TABLE III

Urethanes in Dextrose

The narcotics in the molar concentrations shown are dissolved in 0.38 molal solution of dextrose. In the top row is given the permeability of cells in pure dextrose solution and in the bottom row the permeability in dextrose solution containing 0.01 m. $CaCl_2$. Each permeability value in this experiment is based on measurements of ten cells. The temperature was 12° ± 0.5° C.

It is seen that the narcotics cause a definite decrease in permeability which, however, is not as great as the decrease effected by calcium.

Solution	Permeability		
Dextrose 0.38 m	0.096	0.097	0.092
Dextrose 0.38 + n-butyl carbamate 0.025 m	0.062		
Dextrose 0.38 + i-amyl carbamate 0.01 m		0.085	
Dextrose 0.38 + phenyl urethane 0.0025 m			0.070
Dextrose 0.38 + $CaCl_2$ 0.01 m	0.041	0.047	0.041

of the narcotized cells lie about midway. A number of similar experiments gave the same results. In every case exposure to the narcotic caused a definite decrease in permeability, which was never of the magnitude of the decrease effected by calcium. The conclusion may be drawn that narcotics tend to decrease cell permeability to water but that their effect may be masked by the presence of cations in the medium.

DISCUSSION

Most studies of the effect of narcotics on cell permeability have been concerned only with permeability to various substances in solution.[9] The most important investigations on permeability to water, as influenced by narcotics, are those of Winterstein (1916), Lillie (1918), Heilbrunn (1925) and Anselmino (1928).

Winterstein in his first group of experiments used sartorius muscle of frogs, employing the usual method of weighing. He found that in hypotonic solution of sodium chloride containing alcohol in narcotic concentration the weight increase of muscle was less than in the same

[9] The literature on this subject is reviewed by Gellhorn, E., Das Permeabilitätsproblem, Berlin, 1929; Winterstein, H., Die Narkose, Berlin 1926; Lillie, R. S., Protoplasmic Action and Nervous Action, Chicago, 1924; Bayliss, W. M., Principles of General Physiology, 4th edition, London, 1924; Osterhout, W. J. V., Injury, Recovery, and Death in Relation to Conductivity and Permeability, Philadelphia and London, 1922; Jacobs, M. H., in Cowdry, E. V., General Cytology, Chicago, 1924; Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926; von Tschermak, A., Allgemeine Physiologie, I, Berlin, 1924.

hypotonic solution containing no narcotic. In later experiments Winterstein constructed artificial "cells"; glass cylinders were covered with the thin abdominal muscle of female frogs. It was found that, in agreement with the experiments on sartorius muscle, four different narcotics effected a marked decrease of the water intake by the "cell." The effects were reversible.

Lillie investigated the effect of narcotics on permeability to water of fertilized eggs of *Arbacia*. He had previously shown that in the *Arbacia* egg fertilization is followed by an approximately four-fold increase in permeability to water. This increase of permeability was found to be inhibited by various organic anesthetics. In all cases eggs which were caused to shrink, two or three minutes after insemination, in solutions of these compounds in sea water of the appropriate conconcentrations, remained in the condition of low permeability characteristic of the unfertilized egg. This effect of narcotics was readily reversible.

Heilbrunn repeated Winterstein's experiments, studying the increase in weight of frog's gastrocnemius muscle in distilled water, and in distilled water to which two per cent by volume of ether had been added. He found that water entered etherized muscle somewhat less rapidly than normal muscle. In further experiments Heilbrunn investigated the rapidity of swelling of unfertilized eggs of *Arbacia* in hypotonic sea water containing one or two per cent of ether. His curves show that the cells swelled even more readily in the presence of ether than in its absence. From these experiments Heilbrunn concluded that ether does not lower the permeability of sea urchin eggs to water.[10]

Very recently, Anselmino investigated the effect of various narcotics on permeability to water of dried collodion and of copper ferrocyanide membranes, and found, in agreement with the work of Winterstein on living membrane, a marked decrease of permeability to water.

From the experiments summarized above (excepting Heilbrunn's experiment on *Arbacia* eggs) the conclusion was drawn that narcotics decrease permeability to water. It would appear, however, that the chemical composition of the medium is a factor of importance when investigating the action of narcotics on cell permeability. The experiments reported in the present paper indicate that narcotics, at least in the case of the unfertilized egg of *Arbacia*, do not lower permeability to water when they are caused to act in sea water, or in a medium containing calcium.

[10] It is not improbable that the more rapid swelling of the narcotized cells in Heilbrunn's experiments is due to injury.

Summary

1. The effect of narcotics (urethanes and carbamates) on cell permeability to water was studied by measuring the rate of swelling of unfertilized eggs of the sea urchin, *Arbacia punctulata*, in hypotonic sea water and in hypotonic dextrose solution.

2. Narcotics in the presence of sea water do not decrease permeability to water beyond the value normally found in sea water.

3. But narcotics have a tendency to reduce permeability to water, being, however, less effective in this respect than are bivalent cations. This tendency to decrease permeability is demonstrated when narcotics are used in solutions free from bivalent cations, *i.e.*, in hypotonic solutions of dextrose.

4. The effect of narcotics on permeability to water depends on the chemical composition of the medium in which the narcotizing compound is dissolved.

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THE CHANGES DURING DESICCATION AND REHYDRATION IN THE BODY AND ORGANS OF THE LEOPARD FROG (*RANA PIPIENS*)

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On account of the fundamental importance of water in the living organism, it is of interest to know how much water the body can lose from its storage depots and yet survive. The further question arises as to whether there is, after recovery from desiccation, a normal redistribution of the water in the body as shown by the water content of the various organs. Dehydration is difficult to produce in the mammalian organism, but is easily accomplished in the frog. This animal was therefore used in the present experiments.

Material and Methods

Forty common leopard frogs (*Rana pipiens*) were used in the experiments. They were all males of similar weight and obtained from the same location in southern Minnesota. They were caught in October, and the experiments ran from October through February. The frogs were kept in large concrete tanks through which ran fresh, cold tap water at a temperature ranging from 8° to 11° C. The water was kept at a depth of six inches. Bricks were put into the tanks in such a manner that when the frogs rested on them, only their heads would be exposed. The frogs had reached a constant body weight before being used.

The forty animals were divided into four groups of ten frogs each. These were designated as control group A (controls for normal fresh organ weights); control group B (controls for normal total water content); test group C (frogs desiccated for organ weights), and test group D (rehydrated for organ weights). The frogs in control group A were anesthetized with ether and autopsied at once. Their fresh and dry organ weights were determined. The control group B were killed by the ether and their fresh and dry weights were determined for the body as a whole. The test group C were desiccated so as to lose forty per cent or more of their total body weight, then anesthetized and autopsied to obtain the fresh and dry organ weights. The test group D were desiccated to the same extent as group C, and then

allowed to recover for ten days in water at room temperature of 25° C. (range 20–28° C.). They were then anesthetized and autopsied to secure the fresh and dry organ weights. The body weights of test groups C and D were taken at frequent intervals (see Fig. 1) to determine the rate of dehydration, and, in the case of group D, also the rate of rehydration.

The frogs were desiccated by exposing them to evaporation, each in a cylindrical glass jar 8 inches in height and width. These jars were covered by a metal screen with three meshes to the inch. They were placed in a room, the temperature of which was kept fairly constant by means of a thermostat. The average temperature was 25° C. (range 23–26° C.). The average relative humidity was 25 (range 20–29), measured with a Tycos hygrometer.

Before weighing in all groups, the excess of moisture was removed from the frogs by carefully blotting with paper towels. No allowance was made for the contents of the urinary bladder, as the frogs usually voided urine during the manipulation incident to drying them.

In all cases, ether was used to anesthetize the frogs. With the exception of control group B, the (anesthetized) animals were killed by cutting open the tip of the cardiac ventricle and suspending the animals by the head and feet so that all the blood possible might drain out into a bottle. The weight and the water content of the escaped blood were determined.

The procedure at autopsy was as follows: The head was severed from the body at the atlanto-occipital articulation. The limbs were separated at the hip and shoulder joints, respectively. All parts were placed in a moist chamber until dissected. The dissection was made upon dampened blotting paper. The lungs were removed at the junction with the trachea, and were opened to remove any parasitic flukes found there. The heart was removed by cutting the large veins at their termination, and severing the aorta at its origin from the bulbus aortae. The chambers of the heart were opened and their contents (if any) removed. The contents of the stomach and intestines were removed before weighing these organs. The ano-cloacal junction was taken as the lower border of the intestines. The suprarenal glands were not removed from the kidneys. The liver was removed and weighed together with the gall bladder. The bile in all cases was allowed to drain out before the weighing. The tongue was separated by cutting the muscles at their attachment to the hyoid bone. When the integument was taken, the subjacent lymph was merely allowed to drain off. The term "remainder" includes the various items such as large blood vessels, nerves, connective tissue and other structures not

included under the other headings. The remainder does *not* include the water in the subcutaneous lymph spaces.

All organs were weighed on a Wilkens-Anderson analytical balance in previously weighed weighing bottles with ground glass stoppers. The organs, and likewise the entire frogs of control group B, were dried in a Thelco electric oven at about 95° C. until constant weights were obtained.

Results

General Observations

The frogs of all groups were healthy and normal in all respects. The average and range of initial body weights in grams were as follows: control group A, 43.59 (35.0 − 53.2); control group B, 40.64 (34.5 − 41.6); test group C, 43.57 (37.0 − 53.1); test group D, 44.80 (42.7 − 49.0). It will be noted that in average body weight groups A and C were nearly identical, and group D differed from these by about only two per cent. In the calculations, it is assumed that the initial organ weights were equal in these three groups.

It required 28.6 hours, on the average, to desiccate the test group C to an average weight of 24.98 grams, with loss of 42.7 per cent of their initial body weight, or 51.9 per cent of their total water content (Fig. 1).[1] These frogs at first were quite active, but after a few hours of desiccation they remained motionless, apparently almost lifeless. The skin became very dry, and the animals appeared emaciated. The frogs maintained the sitting position, and the joints of the limbs became stiff. In addition to the ten surviving frogs in this group, four others died during the test and were excluded from the experiment.

A period of 31.8 hours was required to desiccate the test group D to an average weight of 25.40 grams, with loss of 43.3 per cent of their average initial body weight or 52.7 per cent of their total water content.[1] Up to this stage, the general appearances and reactions were the same as in the test group C. Upon being put back into water at room temperature (25° C.) for rehydration, the test group D at first remained inactive. After two or three hours, they gradually began to move about, and soon became normal to all outward appearances. After 26.2 hours these frogs had returned to a nearly constant body weight, averaging 36.74 grams (Fig. 1). This weight they maintained, with slight fluctuations, for ten days before being autopsied. This nearly constant body weight averaged about 8.1 grams below the original body weight. Figure 1 shows the general weight curve fol-

[1] These percentages for the estimated losses of water content are slightly too high, since they include (as shown later) a loss of about 0.51 gram in solids during the experiment. The corrected loss of total water content is 50.5 per cent for group C and 51.3 per cent for group D.

lowed by this group during desiccation and rehydration. In addition to the ten surviving frogs in this group, five others died during the test and were excluded from the experiment. These five frogs died during the dehydration part of the experiment. No deaths occurred during rehydration.

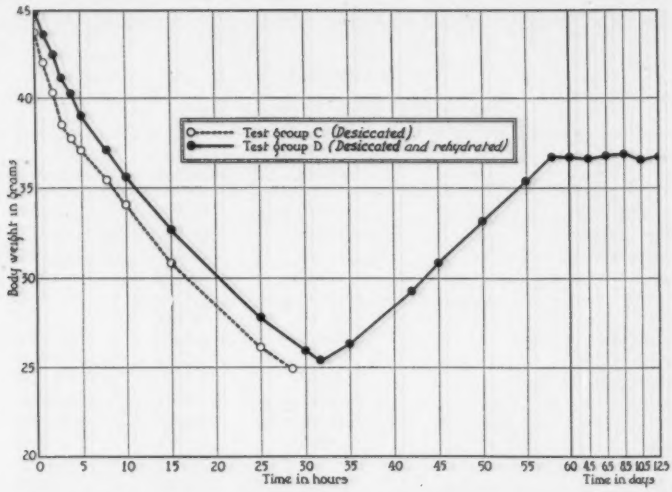

FIG. 1. Curves of average weight during desiccation and rehydration. Test group C was desiccated 28.6 hours, decreasing in average body weight from 43.6 grams to 25.0 grams. This corresponds to a loss of about 43 per cent in body weight. Test group D was similarly desiccated 31.8 hours, decreasing from 44.8 grams to 25.4 grams, with loss of about 43 per cent in body weight. This group was then replaced in water and increased to 36.7 grams in 26.2 hours (end of 58th hour of experiment). Thereafter the weight remained nearly stationary during a period of 10 days. Failure to reach the original weight is ascribed to the increase in room temperature.

Observations on Individual Organs and Parts

Observations at Autopsy.—At autopsy the control group A appeared normal in every respect with the exception of an occasional lung fluke. The test groups C and D also occasionally contained one to three flukes. In the desiccated test group C the blood was so thick that it would barely run into the container. There seemed to be practically no water in the tissue spaces and lymph sacs. The intestinal contents were very slight. Most of the organs appeared very dry.

The rehydrated test group D at autopsy appeared quite normal with the exception that the epidermis was shedding in large pieces quite generally over the body. This process resembled the normal

ecdysis, although in this case it was caused by the desiccation. The amount of water in the tissue spaces and lymph sacs appeared somewhat below that in the normal control group A. The blood seemed normal in color and consistency.

Fresh Weights of Organs.—(Tables I and II.)—The average fresh organ weights of control group A were taken as norms, and the average fresh organ weights of test groups C and D were compared with these.

TABLE I

Average Fresh Organ Weights in Grams

	Control group A (normal)	Test group C (desiccated)	Test group D (rehydrated)
Eyeballs	0.3980	0.3958	0.3987
Lungs	0.2146	0.1992	0.2134
Skeleton	5.2250	5.0073	5.1246
Brain	0.1013	0.0934	0.1093
Kidneys	0.1381	0.1151	0.1442
Heart	0.1112	0.0922	0.1057
Spinal cord	0.0636	0.0498	0.0591
Testes	0.0424	0.0314	0.0456
Stomach-intestines	0.6182	0.4326	0.5812
Muscles	16.5533	11.4997	16.0331
Skin	5.7809	3.3976	4.7196
Tongue	0.3993	0.1892	0.3800
Spleen	0.0381	0.0170	0.0296
Liver	1.6586	0.7019	1.0137
Blood	1.4274	0.2739	1.3468
Fat bodies	0.0950	0.0251	0.0463
Remainder	0.7194	0.4448	0.7808
Total	33.5844	22.9660	31.1317

As shown by Tables I and II, in the test group C, the eyeballs and lungs remained nearly unchanged in weight, and the skeleton and brain lost less than ten per cent through desiccation. The kidneys, heart, spinal cord, testes, stomach-intestines and muscles form a group losing moderately (16–30 per cent), but relatively less than the body as a whole. Finally the skin, tongue, spleen, liver and (escaped) blood lost 43–81 per cent, which exceeds the relative loss of the body as a whole.

In the rehydrated test group D, all the fresh organs, with the exception of the skin, spleen and liver, had regained practically normal weight (within 8 per cent). The skin remained about eighteen per cent below the norm, the spleen 22 per cent below, and the liver nearly 39 per cent below. The significance of these changes will be considered later.

TABLE II

Average Percentage Difference in Fresh Organ Weights of Test Groups C and D, Compared with Control Group A

	Test group C (desiccated)	Test group D (rehydrated)
1. *Organs with slight loss during desiccation*		
Eyeballs	−0.6	±0.0
Lungs	−0.6	−7.2
Skeleton	−4.2	−1.9
Brain	−7.8	+7.9
2. *Organs with moderate loss during desiccation*		
Kidneys	−16.7	+4.4
Heart	−17.1	−5.0
Spinal cord	−21.7	−7.1
Testes	−26.0	+7.6
Stomach-intestines	−30.0	−6.0
Muscles	−30.5	−3.1
3. *Organs with great loss during desiccation*		
Skin	−42.8	−18.4
Tongue	−52.6	−4.8
Spleen	−55.4	−22.3
Liver	−57.7	−38.9
Blood	−80.8	−5.6

Water Content.—(Tables III and IV.)—The average percentage water content found in the whole body of control group B was 82.14 per cent, ranging from 80.21 to 84.28 per cent. This average is taken as the norm for the body as a whole.

The average percentages of water found in the organs of control group A, and of test groups C and D, are given in Table III. For the normal control group A, the organs are arranged in order of increasing relative water content, from the skeleton (58.13) to the escaped blood (88.97 per cent). The muscles in this group contain almost exactly the same percentage of water as the average for the whole body. The skeleton, liver, spinal cord, skin and spleen contain relatively less, and the testes, "remainder," brain, heart, kidneys, stomach-intestines, tongue, lungs, eyeballs and blood contain relatively more water than the body as a whole.

In the desiccated test group C, the organs listed (Table III) have all lost part of their water and hence appear lower in relative water content than the corresponding organs of control group A. Assuming that all the loss of weight in this desiccated group C (Table I) is due to loss of water, we may estimate the theoretical water content of the organs in group C. This theoretical water content should agree

fairly with that actually found in the desiccated test group C (Table III). The calculations of the theoretical water content were made in this way for every organ, but the figures in detail are omitted for economy of space. The muscles on this basis show the closest agreement with a discrepancy of only 0.2 per cent.[2] Agreement (within 3.5

TABLE III

Average Percentage of Water in Organs

	Control group A (normal)	Test group C (desiccated)	Test group D (rehydrated)
Skeleton	58.13	57.94	60.52
Liver	76.08	72.10	76.41
Spinal cord	80.75	76.73	81.67
Skin	81.05	70.84	80.15
Spleen	82.13	77.84	81.39
Muscles	82.15	74.52	82.17
Testes	82.57	75.09	81.40
Remainder	83.12	77.19	81.85
Brain	83.90	81.28	85.09
Heart	84.07	82.24	87.17
Kidneys	84.42	78.09	84.06
Stomach-intestines	85.00	75.21	83.36
Tongue	86.13	76.83	87.87
Lungs	87.11	82.52	86.15
Eyeballs	87.83	85.38	88.16
Blood	88.97	77.33	88.79

per cent) is also found in the case of all the other organs except the liver, spleen, tongue, lungs and skin. The percentage of water in the liver of the desiccated group averages 28.7 per cent greater than that to be expected from its loss of weight. The corresponding percentages in the spleen, tongue, lungs and skin are, respectively, 17.9, 6.8, 4.5 and 4.0 per cent greater than those theoretically calculated from the change in organ weight. The significance of these discrepancies will be discussed later.

The absolute amount of water given up by each of the various organs or parts in test group C is shown in Table IV. The amounts

[2] The method of calculation is as follows: As shown in Table III, every 100 grams of fresh muscle in the normal control group A contains 82.15 grams of water and (accordingly) 17.85 grams of solids. Table II shows that in the desiccated test group C each 100 grams of muscle had lost 30.5 grams in weight, which is assumed to be due to loss of water. Subtracting 30.5 from 82.15 gives 51.65 grams of water remaining in the 69.50 grams of muscle in the desiccated frog. This gives a theoretical water content of 74.32 per cent. The actual water content, as found in the oven-dried muscle of the desiccated test group C (Table III), was 74.52 per cent, which differs from the theoretical value by only 0.2 per cent.

are estimated from the differences in relative water content between groups A and C (Table III), assuming that the initial organ weights were equal to those in control group A. The estimate for the lymph spaces is explained later. The chief storage depots of water in the frog's body are apparently the lymph spaces, yielding 8.51 grams (or

TABLE IV

Organs and Parts of Test Group C, arranged according to the Absolute Amount of Water in Grams Lost During Desiccation. Estimated on basis of change in percentage of water content shown in Table III and normal fresh organ weights in Table I.

Lymph spaces	8.5100	Fat bodies	0.0294
Muscles	5.0289	Lungs	0.0225
Skin	2.2794	Kidneys	0.0210
Blood	1.0582	Spleen	0.0181
Liver	0.7559	Heart	0.0177
Remainder	0.2547	Eyeballs	0.0134
Stomach-intestines	0.2001	Spinal cord	0.0132
Tongue	0.1985	Testes	0.0112
Skeleton	0.1363	Brain	0.0091
		Total	18.5776

45.8 per cent of the total 18.5776 grams of water lost by the body during desiccation); and the muscles, yielding 5.03 grams (or 27.1 per cent). Somewhat smaller contributions are made by the skin (2.28 grams or 12.3 per cent), and the blood (1.06 grams or 5.7 per cent). All the other organs together give up only 1.70 grams, or 9.2 per cent.

In the frogs of the rehydrated test group D, the organs in general have regained nearly normal water content (Table III). All the organs average within 2 per cent of the normal water content found in control group A, excepting the skeleton, which is about 2.4 per cent above normal, and the heart, which averages 3.1 per cent above.

To determine the amount of water in the lymph spaces of control group A, the total average fresh organ weight (33.58 grams, Table I) was subtracted from the average original body weight (43.59 grams). This gives a difference of 10.01 grams, due chiefly to the contents of the lymph spaces. During desiccation, the test group C frogs lost 18.59 grams in average body weight. The organs of this group (excluding the lymph spaces) apparently gave up 10.07 grams of water, as shown by the estimates in Table IV. The remainder of the loss (8.52 grams) was assumed to come from the lymph spaces, although a small part of this loss is in solids, as will be shown later. It is assumed that the lymph spaces of test groups C and D originally contained as much water as the lymph spaces of control group A (10.01 grams). There-

fore, at the time of autopsy the lymph spaces of test group C should theoretically contain 1.49 grams (10.01 minus 8.52 grams). If we subtract the average fresh organ weight of this group (22.97 grams, Table I) from their average final body weight (24.98 grams), we find that the actual amount of water in the lymph spaces was 2.01 grams. The apparent loss of solids in the various organs can be calculated by comparing the theoretical losses of water (Table IV) with the decreases in fresh weight (Table I). All the organs excepting the stomach-intestines, lungs, eyeballs and testes show variable (usually small) losses in weight beyond those due to apparent decrease in water content. The net decrease amounts to about 0.58 gram, or apparently slightly more than sufficient to account for the difference between the theoretical and the actual amount of water in the lymph spaces (0.52 gram).

The total theoretical amount of water in the body, as a whole, in the rehydrated test group D at time of autopsy was 30.18 grams. This is obtained from the average final body weight (36.74 grams) multiplied by the normal percentage of water in the whole body (82.14 per cent). If we subtract the total water actually found in the organs (24.46 grams) from the total (theoretical) water content of the body (30.18 grams), we get the theoretical amount of water in the lymph spaces (5.72 grams) of the rehydrated test group D. We can check this by subtracting the average fresh organ weight of test group D (31.13 grams, Table I) from the average final body weight for this group (36.74 grams). This gives 5.61 grams as the amount of water found in the lymph spaces, which is very close to the theoretical expectation.

In general, therefore, it appears that the lymph spaces of the desiccated test group C had given up approximately 80 per cent of their initial water content, while the lymph spaces of the rehydrated test group D had not fully refilled, but were still about 44 per cent below their estimated original water content. The explanation for this difference will be discussed later.

Discussion

In general, it must be remembered that the number of frogs (10) in each of the four groups is relatively small. The results may therefore be influenced somewhat by individual variations, so that in some cases the conclusions are to be regarded as tentative rather than final.

General Observations

The average loss of weight (about 43 per cent) sustained by desiccation in our test groups C and D may be compared with the results of

other investigators. Chossat (1843) noted a loss of 35 per cent in the body weight of frogs subjected to evaporation. Similar observations were made by Durig (1901). Hall (1922) subjected various species of animals to exsiccation, and found that frogs survived a desiccation period of 33 hours, with loss of 41 per cent in body weight, corresponding to 50 per cent of the entire water content. De Almeida (1926) reported that in frogs the loss in body weight before death from dehydration varies with the rapidity of evaporation. With rapid desiccation, death may occur with a loss of only 10 per cent, while with slower experiments the loss may be 30 to 40 per cent. Further data on dehydration in the frog are given by Iizuka (1926) and Hug (1927). Data for comparison as to storage and loss of water in other species and in other forms of inanition are cited by Jackson (1925, 1929). These include the effects of exsiccation on various invertebrates and vertebrates.

The curves of body weight during dehydration in our test groups C and D (Fig. 1) show a more rapid loss at the beginning, with a progressively slower rate of decrease later. The rate of rehydration followed by test group D appears slower at first, but is more nearly uniform from the third to the 26th hour (58th hour of the experiment), when the weight ceased to increase. This group, therefore, did not return to their original body weights, but maintained a nearly constant body weight averaging 36.74 grams, or 8.06 grams below their original weight. This difference is apparently due chiefly to the partially unrestored loss of water from the lymph spaces. It is probably caused by the change in room temperature, which was 8° to 11° up to the beginning of the experiment, but 25° C. during the test. Ott (1924) found that frogs on transfer to cooler environment may absorb water (chiefly in the lymph spaces), which is lost within a few days when they are removed to warmer quarters.

Individual Organs and Parts

Fresh Weights.—The losses in fresh weights of the organs in the desiccated group C agree in general with the findings of Durig (1901). He reported that the musculature lost most heavily, but he did not measure the loss from the lymph spaces, which we find to be even greater. According to Durig, the brain and heart lost relatively less than any other organ; but our data indicate that the eyeballs, lungs and skeleton (organs not studied by Durig) lost relatively less fresh weight than the brain or heart. Durig also found that the kidneys, in spite of a marked decrease in water content, showed a relative increase in weight, which he ascribed to retention of insoluble urinary constitu-

ents. The kidneys in our desiccated group C lost only 16.7 per cent of their fresh weight, so that they gained in relative weight, as did all the other organs losing relatively less than the body as a whole.

Also in other species under conditions of dehydration, certain organs such as the brain, eyeballs, skeleton and heart maintain their organ weights remarkably well (in some species even increasing their weight under these conditions); while other organs such as the skin, muscles, stomach-intestines, liver and spleen lose a large proportion of their fresh weight. (See review of the literature by Jackson, 1925, 1929.) A similar tendency was found by Ott (1924) in the frog during inanition.

Ott found that the excess water absorbed when frogs were transferred to a cooler environment did not appreciably affect the weights of the various organs, excepting the lungs and fat bodies. In the present experiments, all the organs excepting the skin, spleen, liver and fat bodies (as shown in Table I) in the rehydrated group D had returned to nearly normal weight, and all excepting the heart and skeleton (Table III) had returned to within two per cent of the original normal water content. Data for the water content of the fat bodies are lacking however.

Loss of Solids.—A comparison of the figures for fresh organ weights in Tables I and II shows certain discrepancies between the desiccated test group and the rehydrated test group D. All the organs of test group D returned to within 8 per cent of the normal weight, excepting the skin, liver and spleen. As previously mentioned, these discrepancies are attributed chiefly to loss of solids during the experiment. The liver during the brief period of inanition probably lost much of its glycogen content, which could not be restored by the mere addition of water. This would account for the nearly normal relative water content of the liver in spite of its marked subnormality of weight. The skin in test group D started shedding (ecdysis) three to four days after beginning rehydration, which probably accounts for its marked subnormality in weight, although the relative water content had returned to nearly normal. Similarly the spleen may have lost solids (through escape or destruction of cells), which would account for the subnormality in weight in spite of nearly normal water content. The other organs apparently lost but little or no solids, as was noted above.

Water Content.—The average percentage of water content in the organs of our control group A (Table III) is in general agreement with Ott's figures for his normal control frogs. The differences are within 3 per cent, excepting the spleen, which is higher in his series, and the liver, skeleton and stomach-intestines, which are somewhat lower.

In his fasting frogs with loss of 40 per cent in body weight, the percentage of water content is usually increased, in contrast with our desiccated group C showing marked loss in water content.

Table IV indicates that the greatest absolute sources of water during desiccation in the frog are from the lymph spaces, muscles, skin, blood, liver, "remainder," and stomach-intestines, decreasing in the order named. In relative loss, however, the corresponding order is the lymph spaces, blood, skin, stomach-intestines and muscles.

The escaped blood of the test group C contained 77.33 per cent of water, or 11.64 per cent below the water content of the escaped blood in control group A (Table III). Yet the escaped blood of the rehydrated test group D had regained practically the same relative amount of water as the control group A. The markedly thickened blood of the dehydrated frog is therefore rapidly restored to normal. Durig found that the number of red corpuscles per cu. mm. was more than doubled in the frog by desiccation.

After recovery from desiccation, nearly all the organs of the rehydrated group D return to within two per cent of their normal water content (Table III), although insufficient water has been absorbed to restore the original body weight. Ott (1924) similarly noted that the excess water absorbed by frogs at lower temperature did not affect the weight or water content of the organs, excepting the lungs and fat bodies. The only exceptions in our rehydrated series at higher temperature are the skeleton and heart, which gain an excess water content of 2.39 and 3.10 per cent, respectively. The lymph spaces of the test group D, however, are estimated (as previously mentioned) to have remained 44 per cent subnormal in water content. It is evident that the changes in water content of the frog's body with fluctuations of temperature affect the amounts in the interstitial spaces rather than the water content of the tissues and organs.

It is not the purpose of the present paper to compare the findings of other investigators in various species, but it may be noted that, in general, the main storage depots of water in the frog, aside from the subcutaneous lymph spaces, appear to correspond in general to those found in mammals. The amphibian is more tolerant of dehydration, however, and can survive relatively greater losses. Moreover, the water in the amphibian organism is stored in a more labile form, so that it can be more readily removed and restored. Data for comparison are cited by Jackson (1929).

Summary

The principal results of the present investigation may be summarized briefly as follows:

1. Male frogs (*Rana pipiens*) weighing about 44 grams were able to recover after desiccation with average loss of about 43 per cent of their body weight or about 51 per cent of their total water content.

2. The eyeballs and lungs of the desiccated frogs remained nearly unchanged in average fresh organ weight, and the skeleton and brain lost less than 8 per cent. The kidneys, heart, spinal cord, testes, stomach-intestines and muscles formed a group losing moderately (16–30 per cent), but relatively less than the body as a whole. The skin, tongue, spleen, liver and (escaped) blood lost 43–81 per cent, or relatively more than the body as a whole.

3. The average percentage of normal water content in the entire body was 82.14 per cent (range 80.21–84.28) at temperature of 8 to 11° C.

4. In the normal organs the average percentage of water content ranged from 58.13 in the skeleton to 88.97 in the escaped blood.

5. The decreases in percentage of water content in the desiccated frogs averaged approximately as follows: skeleton 0.2, heart 1.8, eyeballs 2.5, brain 2.6, liver 4.0, spinal cord 4.0, spleen 4.3, lungs 4.6, "remainder" 5.9, kidneys 6.3, testes 7.5, muscles 7.6, tongue 9.3, stomach-intestines 9.8, skin 10.2, and escaped blood 11.6.

6. The average absolute amount (grams) of water given up by the various organs or parts of the desiccated frogs was approximately as follows: lymph spaces 8.5, muscles 5.0, skin 2.3, blood 1.1, liver 0.8, "remainder" 0.3, stomach-intestines 0.2, tongue 0.2, skeleton 0.1. The remaining organs each lost less than 30 mgm. of water.

7. In the rehydrated frogs, all the fresh organs with the exception of the skin, spleen and liver regained nearly normal weight (within 8 per cent). In all the rehydrated organs, except the skeleton and heart, the water content returned to within 2 per cent of the norm. However, the amount of water in the interstitial lymph spaces remained 44 per cent subnormal, probably due to the increased temperature of the room.

8. The following peculiarities were noted in the rehydrated organs: The fresh weight of the skin remained 18 per cent subnormal, apparently due to ecdysis. The fresh weight of the spleen was 22 per cent subnormal, probably through escape or destruction of cells. The liver weight remained 39 per cent subnormal, probably through loss of glycogen. The relative water content in each of these three organs in the rehydrated group was found to be within 0.9 per cent of the normal.

9. In general, the main storage depots of the frog, aside from the lymph spaces, appear to correspond in general to those found in mammals. The amphibian is more tolerant of dehydration, however, and

can survive relatively greater losses. Moreover, the water in the amphibian organism is stored in more labile form, so that it can be more readily removed and restored.

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